PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] :
C07K 16/46, A61K 39/395, 38/19, 35/14, G01N 33/68 // C07K 16/28, 16/32, 16/30, 14/33, 16/14, (A61K 39/395, 38:19)

A1

(11) International Publication Number: WO 98/02463

(43) International Publication Date: 22 January 1998 (22.01.98)

(21) International Application Number: PCT/US97/12013

(22) International Filing Date: 10 July 1997 (10.07.97)

(30) Priority Data:
08/678,194 11 July 1996 (11.07.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/678,194 (CIP)
Filed on 11 July 1996 (11.07.96)

(71) Applicant *(for all designated States except US)*: MEDAREX, INC. [US/US]; 1545 Route 22 East, P.O. Box 992, Annandale, NJ 08801-0992 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DEO, Yashwant, M. [US/US]; 3009 Peacock Drive, Audubon, PA 19403 (US). GRAZIANO, Robert [US/US]; 1046 County Road, Route 519, Frenchtown, NJ 08825 (US). KELER, Tibor [US/US]; 30 Park Road, Ottsville, PA 18942 (US).

(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

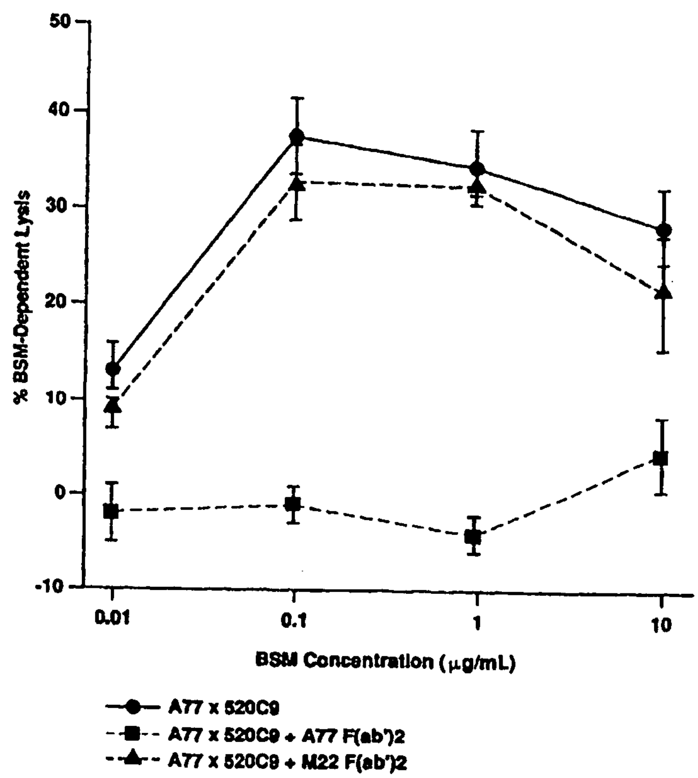
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: THERAPEUTIC MULTISPECIFIC COMPOUNDS COMPRISED OF ANTI-FCα RECEPTOR ANTIBODIES

(57) Abstract

Multispecific compounds comprising at least one binding determinant which binds to the Fcα-receptor on an effector cell. The other binding determinant(s) binds(s) to one or more antigens on a target cell, e.g., the Neu/Her-2 proto-oncogene product or the epidermal growth factor receptor on cancer cells, or to Candida antigens on infected cells. Examples are biospecific and trispecific antibodies. Therapeutic use of said multispecific compounds for treatment of cancers or pathogen infections.



Therapeutic Multispecific Compounds Comprised of Anti-Fcα Receptor Antibodies

Background of the Invention

Receptors for the Fc portions of immunoglobulins are important in triggering many of the protective functions of monocytes, macrophages and polymorphonuclear cells. Receptors for IgG (Fcγ receptors or FcγR) on these cells have been extensively investigated and bispecific molecules targeting these receptors have been constructed. (See e.g. European Patent No. 0 255 249 entitled "Monoclonal Antibodies to Fc Receptor for Immunoglobulin G on Human Mononuclear Phagocytes", which is co-owned by Applicants.) In addition, clinical trials of bispecific molecules (BsAb) which have specificity for the FcγR and the HER-2/*neu* antigen, which is found on breast or ovarian cancers, indicate that these molecules are both safe and efficacious (Valone, Frank H. *et al.* 1995, *J. of Clin. Oncol.* 13(9): 2281-2292).

IgA receptors Fcα receptors (FcαR or CD89) are also capable of promoting effector cell function. Binding of ligand to FcαR triggers phagocytosis and antibody mediated cell cytotoxicity in leukocytes and FcαR-bearing cell lines. Fcα receptors can also cooperate with receptors for IgG on effector cells in enhancing the phagocytosis of target cells. Monoclonal antibodies of the IgM (Shen, L. *et al.*, 1989 *J. Immunol.* 143: 4117) and IgG (Monteiro, R.C. *et al.*,1992 *J. Immunol.* 148: 1764) classes have been developed against FcαR.

IgA is abundant in the human body (Kerr, M.A. 1990, *Biochem. J.* 271:285-296). A single class of IgA Fc receptor, FcαRI or CD89, which binds to monomeric IgA has been identified and characterized (Albrechtsen, M. *et al.*, 1988 *Immunol.* 64:201; Monteiro R., *et al.*, 1990 *J. Exp. Med.*, 171:597). FcαRI is constitutively expressed primarily on cytotoxic immune effector cells including monocytes, macrophages, neutrophils, and eosinophils (Morton, H.C., *et al.*, 1996 *Critical Reviews in Immunology* 16:423). FcαRI expression on a sub-population of lymphocytes (Morton, H.C., *et al.*, 1996 *Critical Reviews in Immunology* 16:423), and on glomerular mesangial cells has been reported (Gomez-Guerrero, C., *et al.*, 1996 *J. Immunol.* 156:4369-4376). Its expression on monocytes and PMN can be enhanced by TNF-α (Gesl, A., *et al.*, 1994 *Scad. J. Immunol.* 39:151-156; Hostoffer, R.W., *et al.*, 1994, *The J. Infectious Diseases* 170:82-87), IL-1, GM-CSF, LPS or phorbol esters (Shen L., *et al., J. Immunol.* 152:4080-4086; Schiller, C.A. *et al.*, 1994, *Immunology*. 81:598-604), whereas IFN-γ and TGF-β1 decrease FcαRI expression (Reterink, T.J.F., *et al.*, 1996, *Clin. Exp. Immunol.* 103:161-166). The α-chain of human FcαRI is a heavily

glycosylated, type one transmembrane molecule belonging to the Ig super-gene family which also includes receptors for IgG and IgE. One gene located on chromosome 19 encodes several alternatively spliced isoforms of the FcαRI alpha chain (55-110 kDa; Morton, H.C., *et al.*, 1996 *Critical Reviews in Immunology* 16:423). Myelocytic FcαRI has been shown to be associated with the FcR γ-chain which is implicated as playing a role in FcαRI signal transduction (Morton, H.C. *et al.* 1995, *J. Biol. Chem.* 270:29781; Pfefferkorn, L.C., *et al.* 1995, *J. Immunol.*, 153:3228-3236, Saito, K. *et al.*, 1995, *J. Allergy Clin. Immunol.* 96:1152).

FcαRI binds both antigen-complexed and monomeric IgA1 and IgA2 (Mazangera, R.L. *et al.*, 1990 *Biochem. J.* 272:159-165), consistent with the receptor being saturated *in vivo* with monomeric IgA in the same manner as FcγR and FcεRI are saturated with IgG and IgE respectively. Cross-linking FcαRI on myeloid effector cells, by polymeric IgA, IgA immune complexes, or mAb specific for epitopes within or outside the ligand binding domain, stimulates degranulation, superoxide release, secretion of inflammatory cytokines, endocytosis and phagocytosis (Patty, C., A. Herbelin, A. Lihuen, J.F. Bach, and R.C. Monteiro. 1995 *Immunology*. 86:1-5; Stewart, W.W., R.L. Maz Yegera, L. Shen, and M.A. Kerr. 1994 *J. Leucocyte Biology*. 56:481-487; Stewart, W.W., and M.A. Kerr. 1990. *Immunology*. 71:328-334; Shen, L. 1992. *J. Leukocyte Biology*. 51:373-378.). These physiological responses triggered via FcαRI can be important in the first line of humoral defense on mucosal surfaces (Morton, H.C., M. van Egmond, and J.G.J. van de Winkel. 1996 *Critical Reviews in Immunology*. 16:423).

Thus FcαRI is a clinically relevant trigger receptor on cytotoxic immune effector cells and its activity can be exploited to develop novel immunotherapies. The cytotoxic potential of FcαRI has not been carefully explored since almost all monoclonal antibody (mAb) based therapies are being developed with mAbs of IgG class which do not bind to FcαRI.

Summary of the Invention

The present invention relates to multispecific therapeutic molecules with binding determinants for immunoglobulin A (IgA) receptors. IgA is the predominant antibody class in fluids on mucosal surfaces, and IgA receptors (Fcα receptors, FcαR or FcαRI) are found on white blood cells including macrophages, monocytes, neutrophils, eosinophils and lymphocytes. The bispecific and multispecific molecules of the invention can be used as therapeutic agents to harness the cytolysis and phagocytosis capabilities of these white blood cells, enhancing the attack of these cells against cancer cells, cells of infectious microorganisms, and cells infected with pathogens.

In one aspect, the invention includes bispecific binding molecules, comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds one or more target antigens. Preferably, the first determinant binds a site on the FcαR that is different from the binding site for endogenous IgA, so that binding of the molecules of the invention is not blocked or is not substantially blocked by IgA. In a preferred embodiment, the target antigen bound by the second binding determinant of the bispecific molecules of the invention is a cancer cell antigen. In a more preferred embodiment the cancer cell antigen is an antigen of a cancer of the breast, ovary, testis, lung, colon, rectum, pancreas, liver, central nervous system, head and neck, kidney, bone, blood or lymphatic system. In another preferred embodiment, the target antigen is an infectious disease antigen from a pathogen or pathogen-infected cell. In yet another embodiment, the invention features treatment of an autoimmune disease with a composition that binds and modulates a receptor for IgA, causing modulation of the receptor such that further binding of IgA to that receptor is decreased. In a different embodiment, the invention provides compositions that bind and do not modulate a receptor for IgA, so that the effector cells of a subject are armed with the bispecific and multispecific molecules and can bind an antigen on a pathogen or on a cancer.

A preferred embodiment of bispecific molecules of the subject invention comprise molecules with binding determinants for a receptor of the human EGF-like receptor family, for a carcinoembryonic antigen, for a gastrin releasing peptide receptor antigen, and for a mucine antigen, which are overexpressed by certain tumor cells.

The bispecific molecules of the invention encompass molecules that are comprised in part of binding determinants of antibodies, and the molecules of the invention include those that are engineered to include at least one antibody or an antibody fragment. The bispecific binding molecules of the invention preferably comprise a binding determinant from an IgG antibody or IgG fragment, including an Fab, Fab', $F(ab')_2$, Fv, and single chain Fv. A binding determinant, including an Fab, Fab', $F(ab')_2$, Fv, and single chain Fv, can be obtained also from an IgA antibody or an antibody of another isotype. A preferred bispecific binding molecule of the invention comprises a first binding determinant that is at least a functional fragment of antibody A77 and a second binding determinant that binds a cancer cell antigen, a pathogen antigen, or an antigen on an infected cell. Other preferred bispecific binding molecules of the invention comprise a first binding determinant that is at least a functional fragment of antibody A3, A59 or A62, which are similar to A77 in affinity to the receptor for IgA and are not blocked by IgA of the subject. The invention includes

nucleic acid sequences encoding the V_H and V_K regions of the A77 antibody and the predicted amino acid sequences of these regions, and these sequence are preferably used for humanizing the A77 binding determinants for therapeutic multispecific molecules. Preferably the second binding determinant of the molecules of the invention is at least a functional fragment of antibody 520C9, antibody H425 or antibody CC49. A preferred embodiment carries one binding determinant for FcαR and one for the HER/*neu* antigen found for example on tumors of the breast, ovary, and lung.

Several methods of producing bispecific binding molecules are encompassed by the invention, including by chemical linkage of the binding determinants, and by recombinant genetic methods. Recombinant bispecific molecules encoded by nucleic acid sequences carrying genes encoding binding determinants which are thus genetically linked are encompassed by the invention. Further, bispecific binding molecules of the invention are produced by cell fusion of two antibody-producing cell lines carrying the respective nucleic acid sequences encoding the binding determinants, such as hybridoma cell lines, to obtain a progeny cell line producing the bispecific molecule of the invention.

In addition to bispecific binding molecules, the instant invention encompasses multispecific binding molecules which comprise at least a first binding determinant which binds an Fcα receptor and a second binding determinant which binds a target antigen, and at least a third binding determinant. Binding of the first determinant of these multispecific binding molecules to FcαR is not blocked or inhibited by human immunoglobulin A, so there is little or no competition for binding by endogenous IgA molecules. Multispecific binding molecules encompass bispecific and trispecific compositions, and those with four or more binding determinants. A preferred embodiment of a trispecific binding molecule carries an additional binding determinant that binds to an Fc receptor that is not an Fcα receptor, including for example a binding determinant for CD2, CD3, Fcγ receptor, Fcε receptor, Fcδ receptor and/or Fcμ receptor, these determinants being in addition to the first binding determinant to Fcα receptor. The most preferred embodiment of an additional binding determinant for an FcR is a determinant for Fcγ receptor. For a multispecific binding molecule of the invention carrying a binding determinant for an Fcγ receptor, binding to FcγR is not inhibited by human IgG, since the molecule binds Fcγ at a different epitopic site from IgG binding of FcγR. By incorporating at least binding determinant for each of FcαR and FcγR into a single molecule, the therapeutic capability of the molecule is increased to enhance affinity and kinetics of binding of white blood cells to tumor cells or cells of pathogenic

organisms or pathogen-infected cells, increasing opportunities for cytolysis and phagocytosis of these targets.

Another preferred embodiment of the multispecific binding molecules with a determinant for Fcα, is a molecule that carries a third binding determinant that binds to a second target antigen or a second target epitope on a cancer cell, a pathogen, or a pathogen-infected cell. The preferred means of producing these molecules is by chemical linkage of the binding determinants, however the invention encompasses also multispecific binding molecules which are recombinantly produced, or which are produced by cell fusion of two or more cell lines each of which carries the nucleic acid sequences encoding the binding determinants. Preferably at least onc binding determinant is an antibody or an antibody fragment, and to improve the success of the outcome during continued treatment of humans, the binding determinant is a humanized antibody, which is engineered to minimize the number of foreign epitopes born by the molecule.

A preferred embodiment of the multispecific binding molecules of this invention is comprised of one or more binding determinants for target cancer cell antigens, particularly cancer cell antigens from breast, ovary, testis, lung, colon, rectum, pancreas, liver, central nervous system, head and neck, kidney, bone, blood and lymphatic system cancers. A different preferred embodiment of the invented multispecific binding molecules comprises, as the target antigen, infectious disease antigens from pathogens or pathogen-infected cells. In one embodiment, the target antigens are infectious disease antigens and antigens expressed on infected cells, for example, antigens from infections by bacteria, fungi, protozoa, and viruses. In a more preferred embodiment, the target antigen is from a pathogenic fungus, including an antigen from a pathogenic yeast. In a most preferred embodiment, the target antigen is from a species of *Candida*, for example, *Candida albicans*.

Suitable targets among cancer cell antigens are preferably members of the human EGF-like receptor family, more preferably the cancer cell antigen is an EGF receptor, and most preferably the cancer cell antigen is HER-2/*neu*, HER-3, HER-4, or a heteromultimeric receptor comprised of at least one HER subunit. Additional preferred cancer cell antigens include carcinoembryonic antigen, gastrin releasing peptide receptor antigen, and TAG 72.

A most preferred multispecific binding molecule comprises at least a first binding determinant that is at least a functional fragment of antibody A77 and a second binding determinant that binds an antigen of a cancer cell, a cell of a pathogenic organism, or a pathogen-infected cell. In preferred embodiments of A77-derived

multispecific binding molecules with a cancer antigen binding determinant, the preferred second binding determinant is at least a functional fragment of antibody 520C9 or antibody CC49. The first binding determinant for an Fcα receptor preferably binds a receptor on a white blood cell. The types of white blood cells to which the molecules bind are preferably macrophages, monocytes, neutrophils, eosinophils, and lymphocytes.

Yet another aspect of the invention comprises multispecific binding molecules in which the molecule includes at least one antigen from a pathogen or pathogen-infected cell, or an antigen from a cancer cell. The molecules of this particular embodiment can serve to deliver these antigens as a vaccine directly to the antigen presenting cells of the immune system to immunize the recipient against an infectious disease or a cancer. These antigens can be taken from known antigenic protein sequences of bacteria, viruses, fungi and protozoans, and from cells infected with these pathogen, or from cancer cells, to immunize the recipient.

The multispecific binding molecules of the invention comprise binding determinants from antibody or antibody fragment molecules which preferably are IgG or IgG fragments. Antibody fragments are preferably Fab, Fab', $F(ab')_2$, $F(ab')_3$, Fv, or single chain Fv as sources of binding determinants for construction of the multispecific binding molecules. Antibody fragments can be obtained from an IgG isotype class of antibody, for example from a hybridoma producing a monoclonal IgG antibody, or from a polyclonal IgG preparation. In a preferred embodiment, a polyclonal IgG preparation is obtained from an animal that has been immunized with an antigen from a pathogenic organism, more preferably from a pathogenic fungus.

Another feature of multispecific binding molecules in which the first binding determinant binds FcαR and the second binding determinant binds an antigen of a target cell, encompasses a third binding determinant which binds to a different antigen on the same target cell as the second binding determinant. Further, embodiments encompass a third binding determinant which binds to a different epitope on the same target antigen as the second binding determinant. These determinants provide a two-fold binding capacity of the multispecific molecule to the target to link it to an immune effector cell, for cytolysis and phagocytosis.

The invention also provides a method for eliminating an unwanted cell in a subject, comprising administering to the subject a therapeutically effective dose of a multispecific binding molecule, which comprises at least a first binding determinant which binds an Fcα receptor and a second binding determinant which binds an antigen on the unwanted cell, in a pharmaceutically acceptable carrier. For human subjects, the binding determinants derived from an antibody can be humanized. Further, therapeutic

treatment can be monitored by obtaining a biological sample from the subject during the course of treatment. Another embodiment of the method for eliminating unwanted cells in a subject involves treating the subject in addition with an agent that enhances the number or activity of Fcα receptors, for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment with the invented composition treatment include at least one of G-CSF, GM-CSF, IFN-γ, and TNF, and protocols involving treatment with the molecules of the subject invention and more than one additional therapeutic agent are envisioned.

In another embodiment, the effector cells of the subject can be armed against an antigen, by administering to the subject a therapeutically effective dose of a multispecific binding molecule, which comprises at least a first binding determinant which binds an Fcα receptor and a second binding determinant which binds the antigen, in a pharmaceutically acceptable carrier. The effector cells thus armed with the multispecific molecule do not modulate or down-regulate the Fcα receptors on their surface, and are capable of binding to a target antigen. For human subjects, the binding determinants derived from an antibody can be humanized. Further, therapeutic treatment can be monitored by obtaining a biological sample from the subject during the course of treatment.

Another preferred embodiment of the invention is a method for eliminating an unwanted cell in a subject, comprising obtaining an aliquot of a sample of blood or blood cells from said subject, treating said blood or blood cells *ex vivo* with a therapeutically effective dose of a multispecific binding molecule of the invention in a pharmaceutically acceptable carrier, said binding molecule comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds one or more target antigens, and returning said treated blood or blood cells to the subject. Preferably, the cells of the sample of blood are isolated and expanded in culture, and more preferably, the cells of said sample of blood are treated with agents that enhance the number or activity of Fcα receptors. For human subjects, the binding determinants derived from an antibody can be humanized. Further, therapeutic treatment can be monitored by obtaining a biological sample from the subject during the course of treatment.

In an aspect, the invention provides a method for treatment of a subject with an infectious disease, comprising administration to the patient of a therapeutically effective dose in a pharmaceutically acceptable carrier of a multispecific binding molecule, wherein a first binding determinant binds an Fcα receptor and a second

binding determinant binds a target antigen from a pathogen or a pathogen-infected cell, enhancing the capacity of the immune system to eliminate the infection.

In yet another embodiment, the invention provides a method for immunizing a subject against a cancer antigen or an antigen found on a pathogen or a cell infected by a, comprising administration in a pharmaceutically acceptable carrier of a composition of a multispecific binding agent bearing one or more antigens of a pathogenic infectious organism, or of an antigen of infected cells, or of a cancer cell. A preferred embodiment of an infectious organism is a pathogenic fungus, including a pathogenic yeast; a more preferred embodiment is a species of *Candida*. For human subjects, the binding determinants derived from an antibody can be humanized. Further, therapeutic treatment can be monitored by obtaining a biological sample from the subject during the course of treatment.

The invention provides also a method for identifying for an agent which modulates Fcα receptors on the surface of cells, involving contacting a sample of cells carrying Fcα receptors with the agentA method for identifying for an agent which modulates Fcα receptors on the surface of cells, comprising contacting a sample of cells carrying Fcα receptors with the agent and determining Fcα receptor activity in the sample with the agent, and in a control sample with an antibody that modulates Fcα receptors such as antibody A77, and in another control sample with cells not contacted with said agent or with antibody; then Fcα receptor activities in the samples are compared, such that a sample of cells contacted with the agent having statistically significant less Fcα receptor activity than control cells not contacted with agent, or having statistically significantly as low Fcα receptor activity as cells in a sample with the antibody, identifies an agent which modulates Fcα receptors on the surface of cells.

In another embodiment, the invention provides a method for designing an agent which modulates Fcα receptors for treatment of autoimmune disease by obtaining a three dimensional model of the A77 anti-Fcα receptor binding site using the sequence determinants of A77 heavy and light chain variable regions. This method involves comparing the amino acid residues of the A77 variable region with that of heavy and light chain variable regions of antibodies of known three dimensional structure, determining placement of non-homologous amino acid residues within the main peptide chain of the binding region of the V_H and V_κ sites, such that the size, shape and charge of the A77 anti-Fcα receptor binding site is determined, screening a library of molecules to obtain those of suitable size, shape and charge by computer modeling that are mimetics of the A77 binding site, and screening such candidates of appropriate size,

shape and charge for activity as potential modulators of Fcα receptors. such that an agent which modulates Fcα receptors is designed.

Brief Description of the Drawings

Figure 1 is a gel filtration HPLC elution profile of a A77X520C9 bispecific molecule preparation, in which the peak at 9.86 min. (~150KD) represents a F(ab')3 heterocomplex comprising one A77 F(ab') and two 520C9 F(ab') molecules, and the peak at 10.43 min. represents a F(ab')2 heterocomplex comprising one F(ab') molecule each of A77 and 520C9; the gel filtration HPLC was performed using a TSK-3000 column.

Figure 2 is a graph showing the extent of binding of A77X520C9. an anti-FcαRXanti-HER2/*neu* bispecific antibody (BsAb), as a function of concentration in μg/ml. to neutrophils (PMN, open squares) and to monocytes (closed squares). in which mean fluorescence intensity analyzed by FACScan is the measure of binding.

Figure 3 is a cytometric analysis showing the extent of binding of A77X520C9, an anti-FcαRXanti-HER2/*neu* BsAb (at 10 μg/ml, final concentration) to effector cells in heparinized whole blood, after incubation for one hour at 0°C; phycoerythrin-anti mouse IgG was added, erythrocytes were lysed, and samples were analyzed by FACScan; data show substantial binding to monocytes (middle panel) and neutrophils (bottom panel), but not lymphocytes (top panel).

Figure 4 is a graph showing binding of A77X520C9, the anti-FcαRXanti-HER2/*neu* BsAb (diamonds), and binding of another BsAb, MDX210. also carrying a determinant for HER2/*neu* (open squares), to target SKBR-3 breast tumor cells, as a function of BsAb concentration in μg/ml. in which mean fluorescence intensity analyzed by FACScan is the measure of binding.

Figure 5 is a graph showing binding to target SKBR-3 breast tumor cells of a further preparation of A77X520C9, an anti-FcαRXanti-HER2/*neu* BsAb. as a function of concentration in μg/ml; SKBR-3 cells were incubated with various concentrations of A77X520C9 (circles), A77 F(ab')2 (triangles), or 520C9 F(ab')2 (squares) on ice; cells were washed, stained with anti-mouse IgG conjugated with fluorescein iso-thiocyanate (FITC), and analyzed by FACScan.

Figure 6 is a graph showing A77XH425 BsAb binding to A431 target cells overexpressing EGF-R, in which mean fluorescence intensity analyzed by FACScan is the measure of binding; cells were incubated with various concentrations of A77XH425 (circles), A77F(ab')2 (triangles), or with H425 F(ab')2 (squares); cells were

incubated with antibody as indicated on ice, washed, stained with anti-human IgG conjugated with FITC, and analyzed by FACScan.

Figure 7 is a graph showing A77X520C9 BsAb-mediated antibody-dependent cellular toxicity by cell lysis of SKBR-3 breast tumor target cells by neutrophil effector cells, at a ratio of effector to target cells of 200 to one, as a function of BsAb concentration in μg/ml, with two different preparations of BsAb.

Figure 8 is a graph showing A77X520C9 BsAb-mediated antibody-dependent cellular toxicity by cell lysis of SKBR-3 breast tumor target cells by effector cells in whole blood, using independent duplicate BsAb preparations, as a function of BsAb concentration in μg/ml.

Figure 9 is a bar graph showing inhibition by anti-FcαR A77 $F(ab')_2$ of A77X520C9 BsAb-mediated killing of breast tumor cells by monocytes, compared to absence of inhibition by addition of anti-FcγR M22 $F(ab')_2$.

Figure 10 is a graph showing A77X520C9 BsAb-mediated cytotoxicity by lysis of target SKBR-3 cells by effector PMN cells purified from whole blood; target SKBR-3 cells were labeled with 100μCi of ^{51}Cr for 1 hour prior to combining with PMNs and another preparation of BsAb indicated here as "BSM" (circles); and this mixture of target cells, PMNs and BsAb in the presence of 50μg/ml of A77 $F(ab')_2$ (squares); and this mixture in the presence of M22 $F(ab')_2$ (triangles), each in a 'U' bottom microtiter plate; cell and antibody mixtures were incubated for 16 hours at 37°C, supernatants were collected and analyzed for radioactivity, and cytotoxicity was calculated by the formula: % Lysis = (experimental CPM-target leak CPM / detergent lysis of CPM target leak CPM) X100%. BsAb-dependent lysis = % Lysis with BsAb - % Lysis without BsAb, with an effector:target ratio of 200:1; error bars represent +/- the standard deviation of the mean of values obtained from triplicate experiments.

Figure 11 is a graph showing A77X520C9 BsAb-mediated cytotoxicity by lysis of target SKBR-3 cells, using as effector cells monocytes purified from Leukopaks, with an effector:target ratio of 100:1; other conditions and symbols were the same as those in the description of Figure 10.

Figure 12 is a graph showing A77X520C9 BsAb-mediated cytotoxicity by lysis of target SKBR-3 cells, using the effector cells in whole blood, other conditions and symbols were the same as those in the description of Figure 10.

Figure 13 is a graph showing cytoxicity of A431 target cells that overexpress EGF-R, mediated by A77XH425 BsAb (indicated here as "BSM": circles) and PMN effector cells purified from whole blood; cells were incubated also with BsAb in the presence of 50 μg/ml of A77 $F(ab')_2$ (squares) or M22 $F(ab')_2$ (triangles); target

cells were labeled with 100μCi of ^{51}Cr for 1 hour and cytotoxicity was determined as in the brief description of Figure 10.

Figure 14 is a graph showing A77XH425 BsAb mediated lysis of A431 target cells by monocytes purified from whole blood by Leukopaks (E:T ratio 100:1); other conditions and symbols were the same as those in the brief description of Figure 13.

Figure 15 is a graph showing A77XH425 BsAb mediated lysis of A431 target cells by the effector cells in whole blood; other conditions and symbols were the same as those in the brief description of Figure 13.

Figure 16 is a graph showing Anti-TAG 72 x A77 BsAb-mediated antibody-dependent cellular cytoxicity by neutrophils of TAG 72-bearing tumor cells, as a function of BsAb antibody concentration in μg/ml.

Figure 17 is a graph showing stimulation by the BsAb composition A77XTT of growth of tetanus toxoid (TT)-specific T cells, measured by a spectorphotometric assay of cellular lactate dehydrogenase, by monocyte presentation of this antigen by the BsAb, compared to that of uncomplexed TT, as a function of concentration of each antigen in μg/ml.

Figure 18 is a graph showing the extent of A77X520C9 BsAb-mediated phagocytosis, as a function of concentration in μg/ml, of SKBR-3 breast tumor target cells at a ratio of 15 neutrophils per target cell (circles), or 3 monocytes (squares) per target cell.

Figure 19 is a flow cytometric analysis of BsAb-mediated phagocytosis of SKBR-3 cells by monocyte-derived macrophages (MDM); SKBR-3 target cells were labeled with the lipophilic red fluorescent dye, PKH 26, and cultured with MDM in the absence or presence of A77X520C9 BsAb (or control antibodies) at 37°C for 24 hours; MDM and non-phagocytized tumor cells were recovered with trypsin, and were stained with a FITC labeled anti-CD14, and samples were analyzed by two color fluorescence by FACScan. Percent phagocytosis was calculated as the number of dual-positive target cells (ingested by MDM) divided by the total number of target cells.

Figure 20 is a graph showing phagocytosis of SKBR-3 cells with A77X520C9 BsAb, in which specific phagocytosis of SKBR-3 cells was induced by A77X520C9 BsAb (circles) as compared to the presence of both un-coupled $F(ab')_2$ fragments of A77 and 520C9 (triangles). The BsAb-mediated phagocytosis was blocked by the addition of 10 μg/ml A77 $F(ab')_2$ (squares). BsAb-dependent phagocytosis was calculated as: % phagocytosis with BsAb - % phagocytosis without BsAb.

Figure 21 is a photographic and flow cytometric illustration of neutrophil-mediated phagocytosis of the fungal pathogen, *Candida albicans*, in the presence of A77Xanti-*Candida* (A77Xα-*Candida*) BsAb; panels A and B are representative photomicrographs, and panels C and D are FACScan analyses, of neutrophils that were mixed with *C. albicans* cells in the absence (panels A and C) and presence (panels B and D) of the BsAb.

Figure 22 is a graph showing the extent of BsAb-mediated killing, as a function of concentration in μg /ml, of SKBR-3 breast tumor target cells by neutrophils treated with G-CSF (top panel), or with G-CSF and IFN-γ (bottom panel).

Figure 23 is a graph showing the extent of BsAb-mediated killing, as a function of concentration in μg/ml, of SKBR-3 breast tumor target cells by effector cells which are IFN-γ-treated monocytes (top right panel), by TNF-treated monocytes (bottom panel), or by untreated monocytes (top left panel).

Figure 24 is a bar graph showing that pre-incubation of effector cells with human IgA does not affect binding of A77 mAb, compared to a control incubated in the absence of IgA.

Figure 25 is a bar graph showing modulation of Fcα receptors by incubation of cells with A77 mAb at concentrations ranging from 0.001 to 10 μg/ml, showing extent of change in number of cell surface receptors as a function of incubation with increasing A77 concentration, in comparison with control mAb 520C9 at 10 μg/ml.

Figure 26 is a bar graph showing that BsAb binding to monocytes or to PMN effector cells does not mediate FcαR modulation; modulation of FcαR after A77X520C9, A77 F(ab')$_2$ or A77 mAb binding to PMN and monocytes was examined by flow cytometry, with various concentrations of antibodies added directly to whole blood and incubated overnight at 37°C in medium containing 5%CO_2. Erythrocytes were lysed, and the surface level of FcαR expression on PMN and monocytes was determined by incubation with an anti-huIgA-PE probe at 4°C. Modulation was calculated as: [1-(MFI of sample/MFI of no antibody/BSM control)] x 100%.

Figure 27 shows the DNA sequence of the light chain variable V_K region of the gene encoding the A77 anti-FcαR antibody (SEQ ID NO: 5), and the predicted amino acid residue sequence (SEQ ID NO: 6).

Figure 28 shows the DNA sequence of the heavy chain variable V_H region of the gene encoding the A77 anti-FcαR antibody (SEQ ID NO: 7), and the predicted amino acid residue sequence (SEQ ID NO: 8).

Detailed Description of the Invention

I. Definitions

Definitions of the terms and phrases as used herein should have the meanings indicated below. An antibody (or fragment thereof) is used in the invention as a component of multispecific agents which cause association of a cytolytic, phagocytic white blood cell with a tumor cell, or unwanted infectious disease agent or infected cell. Antibodies suitable for use in the methods of the invention are available in the art (e.g., from the American Type Culture Collection, Rockville, MD, or commercially, e.g., from Becton-Dickinson or Immunotech) or can be prepared by standard techniques for making antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active determinants of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen. Structurally, the simplest naturally occurring antibody (e.g., IgG) comprises four polypeptide chains, two copies of a heavy (H) chain and two of a light (L) chain, all covalently linked by disulfide bonds. Specificity of binding in the large and diverse set of antibodies is found in the variable (V) determinant of the H and L chains; regions of the molecules that are primarily structural are constant (C) in this set.

Structurally, a naturally occurring antibody (e.g., IgG, M_r 150 kDa) consists of four polypeptide chains, two copies of a heavy (H) chain and two of a light (L) chain (M_r 25 kDa), the four chains being covalently linked by disulfide bonds. Specificity of antigen binding by each molecule that comprises the large and diverse set of antibodies is found in the variable (V) determinant of the H and L chains: regions of the molecules that are primarily structural are constant (C) in this set. IgA (M_r 160 kDa) is the predominant antibody in secretions (saliva, tears, mild, nasal mucus and gastrointestinal and respiratory secretions). IgA can exist as a monomer, dimer, and higher multimeric forms (J. Kendrew, Ed., The Encyclopedia of Molecular Biology, 1994, Blackwell Science, Oxford). A further isotype is IgM can be found as a membrane-bound monomer (M_r 190 kDa) on B cells, and as a circulating secretory form as a pentamer (M_r ca. 950 kDa) that differs from the bound form at the N-terminus of the heavy chain due to alternative splicing. The heavy chain of the pentamer is attached by disulfide bridges to a J chain molecule. Minor antibody isotypes include IgD (M_r 175 kDa), expressed on cell surfaces, and IgE. IgE (total M_r 190 kDa) comprises 0.0003% of serum immunoglobulin, however it can be substantially elevated in an allergic subject such as an asthmatic, and is medically important as the major mediator of immediate type hypersensitivity.

The binding sites of the proteins that comprise an antibody, i.e., the antigen-binding functions of the antibody, are localized by analysis of fragments of a naturally-occurring antibody. Thus, antigen-binding fragments are also intended to be designated by the term "antibody." Examples of binding fragments encompassed within the term antibody include: a Fab fragment consisting of the V_L, V_H, C_L and C_{H1} domains; an Fd fragment consisting of the V_H and C_{H1} domains; an Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; a dAb fragment (Ward *et al.*, 1989 *Nature* 341:544-546) consisting of a V_H domain; an isolated complementarity determining region (CDR); and an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab' fragments linked by a disulfide bridge at the hinge region. These antibody fragments are obtained using conventional techniques well-known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. The term "antibody" is further intended to include bispecific and chimeric molecules having at least one antigen binding determinant derived from an antibody molecule. Furthermore, although the H and L chains of an Fv fragment are encoded by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain antibody, sAb; Bird *et al.* 1988 *Science* 242:423-426; and Huston *et al.* 1988 *PNAS* 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the term "antibody", and may be utilized as binding determinants in the design and engineering of a multispecific binding molecule. Antibody fragments are also useful for modulating the number of receptors for that antibody on the surface of cells, and for obtaining agents that mimic this activity, by screening for such agents in an assay for modulation of the receptor.

Polyclonal antibodies are produced by immunizing animals, usually a mammal, by multiple subcutaneous or intraperitoneal injections of an immunogen (antigen) and an adjuvant as appropriate. As an illustrative embodiment, animals are typically immunized against a protein, peptide or derivative by combining about 1 μg to 1 mg of protein capable of eliciting an immune response, along with an enhancing carrier preparation, such as Freund's complete adjuvant, or an aggregating agent such as alum, and injecting the composition intradermally at multiple sites. Animals are later boosted with at least one subsequent administration of a lower amount, as 1/5 to 1/10 the original amount of immunogen in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Animals are subsequently bled, serum assayed to determine the specific antibody titer, and the animals are again boosted and assayed until the titer of antibody no longer increases (i.e., plateaus).

Such populations of antibody molecules are referred to as "polyclonal" because the population comprises a large set of antibodies each of which is specific for one of the many differing epitopes found in the immunogen, and each of which is characterized by a specific affinity for that epitope. An epitope is the smallest determinant of antigenicity, which for a protein, comprises a peptide of six to eight residues in length (Berzofsky, J. and I. Berkower, (1993) in Paul, W., Ed., Fundamental Immunology, Raven Press, N.Y., p.246). Antibody affinities for the antigen range from low, e.g. 10^{-6} M, to high, e.g., 10^{-11} M. The polyclonal antibody fraction collected from mammalian serum is isolated by well known techniques, e.g. by chromatography with an affinity matrix that selectively binds immunoglobulin molecules such as protein A, to obtain the IgG fraction. To enhance the purity and specificity of the antibody, the specific antibodies may be further purified by immunoaffinity chromatography using solid phase-affixed immunogen. The antibody is contacted with the solid phase-affixed immunogen for a period of time sufficient for the immunogen to immunoreact with the antibody molecules to form a solid phase-affixed immunocomplex. Bound antibodies are eluted from the solid phase by standard techniques, such as by use of buffers of decreasing pH or increasing ionic strength, the eluted fractions are assayed, and those containing the specific antibodies are combined.

The term "monoclonal antibody" or "monoclonal antibody composition" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody (mAb) composition displays a single binding specificity and affinity for a particular epitope. Monoclonal antibodies can be prepared using a technique which provides for the production of antibody molecules by continuous growth of cells in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497; see also Brown *et al.* 1981 *J. Immunol* 127:539-46; Brown *et al.*, 1980, *J Biol Chem* 255:4980-83; Yeh *et al.*, 1976, *PNAS* 76:2927-31; and Yeh *et al.*., 1982, *Int. J. Cancer* 29:269-75) and the more recent human B cell hybridoma technique (Kozbor *et al.*, 1983, *Immunol Today* 4:72), EBV-hybridoma technique (Cole *et al.*., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96), and trioma techniques.

Tumor specific mAb of human IgA class are not available. Also, it is likely that serum IgA (up to 4.0mg/ml) may interfere with the activity of IgA mAbs under physiological conditions. Another approach employs bispecific antibody molecules to enable FcαRI-dependent cell-mediated cytotoxicity of tumor targets. Bispecific molecules (BsAb) which simultaneously bind to target cells (tumor cells,

pathogens) and a trigger receptor (e.g. CD3, CD2, FcγR) on immune effector cells have been described (Michon, J., *et al.* 1995, *Blood*, 86:1124-1130; Bakács, T., *et al.* 1995, *International Immunology*, 7,6:947-955). BsAbs can be generated from hetero-hybridomas, or by chemically or genetically linking F(ab') fragments of two antibodies with different specificities or a F(ab') fragment and a ligand (Graziano, R.F., *et al.* 1995, *In* Bispecific Antibodies. M.W. Fanger, editor. R.G. Landes Company/Austin, TX; Goldstein, J. *et al.*, 1997 *J. Immunol.* 158:872-879). BsAbs produced using a trigger receptor-specific antibody, that binds outside the natural ligand binding domain of the trigger receptor, can circumvent interference by serum antibodies and recruit immune effector cells in the presence of saturating concentration of the natural ligand (Fanger, M. *et al.*, 1989, *Immunol. Today*, 10,3:92-99). This strategy has been used to produce Fc γR-specific BsAbs, which mediate antibody-dependent cellular cytotoxicity (ADCC) of tumor cells in the presence of monomeric or aggregated IgG (Michon, J., *et al.* 1995, *Blood*, 86:1124-1130; Bakács, T.,*et al.* 1995, *International Immunology*, 7,6:947-955), and have shown promising results in clinical settings, Deo, Y.M., *et al.*, 1997, *Immunol. Today*, 18:127-135. Four FcαRI-specific mAb, identified as A3, A59, A62 and A77, which bind FcαRI outside the IgA ligand binding domain, have been described (Monteiro, R.C. *et al.*, 1992, *J. Immunol.* 148:1764).

A monoclonal antibody can be produced by the following steps. In all procedures, an animal is immunized with an antigen such as a protein (or peptide thereof) as described above for preparation of a polyclonal antibody. The immunization is typically accomplished by administering the immunogen to an immunologically competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained on a booster schedule for a time period sufficient for the mammal to generate high affinity antibody molecules as described. A suspension of antibody-producing cells is removed from each immunized mammal secreting the desired antibody. After a sufficient time to generate high affinity antibodies, the animal (e.g., mouse) is sacrificed and antibody-producing lymphocytes are obtained from one or more of the lymph nodes, spleens and peripheral blood. Spleen cells are preferred, and can be mechanically separated into individual cells in a physiological medium using methods well known to one of skill in the art. The antibody-producing cells are immortalized by fusion to cells of a mouse myeloma line. Mouse lymphocytes give a high percentage of stable fusions with mouse homologous myelomas, however rat, rabbit and frog somatic cells can also be used. Spleen cells of the desired antibody-producing animals are immortalized by fusing with myeloma cells.

generally in the presence of a fusing agent such as polyethylene glycol. Any of a number of myeloma cell lines suitable as a fusion partner are used with to standard techniques, for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines, available from the American Type Culture Collection (ATCC), Rockville, Md.

The fusion-product cells, which include the desired hybridomas, are cultured in selective medium such as HAT medium, designed to eliminate unfused parental myeloma or lymphocyte or spleen cells. Hybridoma cells are selected and are grown under limiting dilution conditions to obtain isolated clones. The supernatants of each clonal hybridoma is screened for production of antibody of desired specificity and affinity, e.g., by immunoassay techniques to determine the desired antigen such as that used for immunization. Monoclonal antibody is isolated from cultures of producing cells by conventional methods, such as ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (Zola *et al.*, Monoclonal Hybridoma Antibodies: Techniques And Applications, Hurell (ed.), pp. 51-52, CRC Press, 1982). Hybridomas produced according to these methods can be propagated in culture *in vitro* or *in vivo* (in ascites fluid) using techniques well known to those with skill in the art.

For therapeutic use of antibodies of non-human origin in humans, the non-human "foreign" epitopes elicit immune response in the patient. If sufficiently developed, a potentially lethal disease known as HAMA (human antibodies against mouse antibody) may result. To eliminate or minimize HAMA, it is desirable to engineer chimeric antibody derivatives, i.e., "humanized" antibody molecules that combine the non-human Fab variable region binding determinants with a human constant region (Fc). Such antibodies are characterized by equivalent antigen specificity and affinity of monoclonal and polyclonal antibodies described above, and are less immunogenic when administered to humans, and therefore more likely to be tolerated by the patient.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the Fc constant region of a murine (or other species) monoclonal antibody molecule is digested with restriction enzymes to remove the region encoding the murine Fc, and the equivalent portion of a gene encoding a human Fc constant region is substituted. (see Robinson *et al.*, International Application WO 87/02671; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., International Application WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567;

Cabilly et al., European Patent Application 125,023; Better *et al.* 1988 *Science* 240:1041-1043); Liu *et al.* 1987 *PNAS* 84:3439-3443; Liu *et al.*, 1987, *J. Immunol.* 139:3521-3526; Sun *et al.* 1987 *PNAS* 84:214-218; Nishimura *et al.*., 1987, *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.*, 1988, *J. Natl Cancer Inst.* 80:1553-1559.)

The chimeric antibody can be further humanized by replacing sequences of the Fv variable region which are not directly involved in antigen binding with equivalent sequences from human Fv variable regions. General reviews of humanized chimeric antibodies are provided by Morrison, S. L., 1985, *Science* 229:1202-1207 and by Oi *et al.*, 1986, *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from 7E3, an anti-$GPII_bIII_a$ antibody producing hybridoma. The recombinant DNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable humanized antibodies can alternatively be produced by CDR substitution U.S. Patent 5,225,539; Jones *et al.* 1986 *Nature* 321:552-525; Verhoeyan *et al.*. 1988 *Science* 239:1534; and Beidler *et al.* 1988 *J. Immunol.* 141:4053-4060).

Human mAb antibodies directed against human proteins can be generated using transgenic mice carrying the complete human immune system rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood *et al.* International Application WO 91/00906, Kucherlapati *et al.* PCT publication WO 91/10741; Lonberg *et al.* International Application WO 92/03918; Kay *et al.* International Application WO 92/03917; Lonberg, N. *et al.* 1994 *Nature* 368:856-859; Green, L.L. *et al.* 1994 *Nature Genet.* 7:13-21; Morrison, S.L. *et al.* 1994 *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman *et al.*. 1993 *Year Immunol* 7:33-40; Tuaillon *et al.* 1993 *PNAS* 90:3720-3724; Bruggeman *et al.*. 1991 *Eur J Immunol* 21:1323-1326).

Monoclonal antibodies can also be generated by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display see e.g., Sastry *et al.* 1989 *PNAS* 86:5728; Huse *et al.* 1989 *Science* 246:1275;

and Orlandi *et al.* 1989 *PNAS* 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick *et al.*,1991, *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick *et al.*, 1991, *Methods: Companion to Methods in Enzymology* 2:106-110).

The term "complement" refers to a set of more than 30 serum proteins that are universally present without prior exposure to a particular antigen (see, Liszewski, M. *et al.*, 1993, Fundamental Immunol., 3*rd* Ed., W. Paul Ed. Ch. 26 "The Complement System" p. 917). The function of the complement system is modification of the membrane of an infectious agent, and promotion of an inflammatory response through cell action. Complement proteins are converted to active forms by a series of proteolytic cleavages. Production of a reactive C3b protein can occur quickly and efficiently via the "classical" complement pathway, or slowly and inefficiently via the "alternative" pathway. C3 is secreted by monocytes and macrophages; a complex of Factors B and D and properdin cleave C3 to yield the products C3a and C3b. These products promote mast cell degranulation, releasing inflammatory molecules such as histamine, proteases, lysozyme, acid hydrolases, and myeloperoxidase. Opsonization of target cell membranes promotes lysis and phagocytosis.

As used herein, the term "cytokine" means a protein hormone that can mediate immune defenses against "foreign" substances or organisms. General properties of cytokines are reviewed, for example, by Abbas, A. *et al.* Cell and Molecular Immunology, 2nd Ed., 1994, Saunders, Philadelphia. Inflammatory cytokines include tumor necrosis factor (TNF), interleukin 1β (IL-1β), IL-6, and γ-interferon (IFN-γ). Production of cytokines by the host can be stimulated by a microbial product, such as lipopolysaccharide (LPS), or by a foreign antigen.

Cytokines can be produced by cells of the immune system, for example, T cells and basophils, and can act on a nearby other cell (paracrine action), or on the producing cell (autocrine action), or can be released into the circulation to act on a distant cell (endocrine). Categories of function of cytokines include: mediation of natural immunity; regulation of lymphocyte activation, growth, and differentiation;

regulation of immune-mediated inflammation; and stimulation of leukocyte growth and differentiation.

Cytokine function is initiated by binding to a specific receptor on a target cell. For example, the 17kD TNF polypeptide which functions as a trimer, is produced by phagocytes and T cells. It binds to a specific TNF-receptor located on, for example, a neutrophil or an endothelial cell to activate the responses of inflammation. One such response in these target cells is production of IL -1β, which in turn provokes production of IL-6. Both TNF and IL -1β act on thymocytes to initiate a signal cascade culminating in increased expression of genes encoding Ig proteins. Similarly, IFN-γ binds to specific cell receptors to stimulate expression of different sequences. These cytokines also bind to receptors on liver cells to activate expression of proteins of the acute phase of immune response.

Other cytokines can be anti-inflammatory in their effects on the immune system, for example, IL-4, IL-10, and IL-13 (Joyce, D. *et al.* 1994, Eur. J. Immunol. 24: 2699-2705; Zurawski, G., *et al.* 1994, Immunol. Today 15: 19-26). IL-10 thus reduces the pro-inflammatory effects of TNF by down-regulating surface TNF receptor (TNF-R) expression, increasing production of soluble TNF-R, and inhibiting the release of TNF.

Further, the function of human IL-13 protein, studied by stimulation of monocytes with LPS, inhibits production of IL-1α, IL-1β, IL-6, IL-8, MIP-1α, TNF-α, IL-10, GM-CSF and G-CSF. Further, production of IL-1ra (receptor antagonist), a soluble form of the IL-1 receptor, is enhanced. These anti-inflammatory properties are similar to those of IL-4 and IL-10.

Immune response to "foreign" antigens comprises the notion that "self" proteins and other molecules expressed within an organisms are not antigenic or immunogenic to that organism. In fact, discrimination between isologous or homologous determinants and foreign, or heterologous determinants is achieved through maturation of the immune system of an organism during development of the immune system. A system of selection against immune cells bearing antibodies with binding determinants to "self" occurs, so that when mature the immune system does not attack proteins or other molecules native to the organism. In certain pathological conditions known as "autoimmune diseases," however, such discrimination is not as accurate, and endogenous structures may be subject to attack from the immune system. Examples of autoimmune diseases and conditions in which there is autoimmune exacerbation of symptoms include systemic lupus erythematosus, myasthenia gravis, multiple sclerosis, and rheumatoid arthritis. Compositions of the instant invention which are capable of binding to a site on the Fcα receptor, by virtue of comprising a binding determinant of

an antibody for a site on this receptor, can also modulate the number of these receptors on the cell surface, and accordingly are potential agents for treatment of autoimmune diseases. Further, amino acid residue sequence data of the Fv regions of the antibody binding determinant is the basis for obtaining a three-dimensional model of the protein features, such as size, charge, and shape of the set of residues which comprise this binding site, so that agents which mimic this binding site may be designed.

The agents of the invention are administered to subjects in biologically compatible forms suitable for pharmaceutical administration *in vivo* to produce a therapeutic response against a cancer or an infectious disease. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects and side effects are outweighed by the therapeutic effects of the composition.

The term "subject," as used herein, refers to a living animal or human in need of susceptible to a condition, in particular a "cancer or infectious disease" as defined below. The subject is an organism possessing leukocytes capable of responding to antigenic stimulation and growth factor stimulation. In preferred embodiments, the subject is a mammal, including humans and non-human mammals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. In the most preferred embodiment, the subject is a human. The term "subject" does not preclude individuals that are entirely normal with respect to cancer, infectious disease, or normal in all respects.

The term "patient," as used herein, refers to a human subject who has presented at a clinical setting with a particular symptom or symptoms suggesting one or more therapeutic regimens. A patient may be in need of further categorization by clinical procedures well-known to medical practitioners of the art (or may have no further disease indications and appear to be in any or all respects normal). A patient's diagnosis may alter during the course of disease progression, such as development of further disease symptoms, or remission of the disease, either spontaneously or during the course of a therapeutic regimen or treatment, or rediagnosis as being entirely of normal condition.

The term "infectious disease" is meant to include disorders caused by one or more species of bacteria, viruses, fungi, and protozoans, which are disease-producing organisms collectively referred to as "pathogens." The term "fungi" is meant to include the yeasts. In this invention, pathogens are exemplified, but not limited to, Gram-positive bacteria such as *Enterococcus fecalis, Hemophilus pneumoniae, Listeria monocytogenes, Mycobacterium tuberculosis, M. leprae. Proprionibacterium acnes. Staphylococcus aureus, S. epidermis, S. intermedias, Streptococcus hemolyticus, S.*

pneumoniae; Gram-negative bacteria such as *Flavobacterium meningosepticum, Helicobacter pylori*, *Hemophilus pneumoniae*, *H. influenzae*. *Klebsiella pneumonia*. *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Shigella dysenteria*, *Salmonella typhi*, *S. paratyphi*, *Escherichia coli* serotype 0157, *Chlamydia* species, *Helicobacter* species; viruses such as HIV-1,-2, and -3, HSV-I and -II, non-A non-B non-C hepatitis virus, pox viruses, rabies viruses, and Newcastle disease virus; fungi such as *Candida albicans*, *C. tropicalis*, *C. krusei*, *C. pseudotropicalis*, *C. parapsilosis*, *C. quillermondii*, *C. stellatoidea*, *Aspergillus fumigatus*, *A. niger*, *A. nidulans*, *A. flavus*, *A. terreus*, *Absidia corymbifera*, *A. ramosa*, *Cryptococcus* neoforms, *Histoplasma capsulatum*, *Coccidioides immitis*, *Pneumocystis carinii*, *Rhizopus arrhizus*, *R. oryzae*. *Mucor pusillus* and other fungi; and protozoa such as *Entamoeba histolytica*, *Entamoeba coli*, *Giardia lamblia*, *G. intestinalis*, *Eimeria sp.*, *Toxoplasma sp.*, *Cryptosporidium parvum*, *C. muris*. *C. baileyi*, *C. meleagridis*, *C. wrairi*. and *C. nosarum*. Obtaining unique epitopes from these organisms by screening proteins and by assaying peptides *in vitro* are commonly known to those skilled in the art.

II. Multispecific molecules

The instant invention relates in one embodiment to recombinant multispecific molecules, which have affinity for and are capable of binding at least two different entities. Multispecific molecules can include bispecific molecules comprised of a binding determinant for an Fc receptor and a binding determinant for a target. The preferred multispecific molecules for the instant invention include molecules which are comprised of at least one copy of a binding determinant which binds specifically to an Fcα receptor or target; or molecules comprised of at least one binding determinant which binds an Fcα receptor, one binding determinant for a target and additionally one or more binding determinants that recognize other molecules. A preferred multispecific molecule is a bispecific antibody (BsAb), which carries at least two different binding determinants, at least one of which is of antibody origin.

A "binding determinant for an Fcα receptor" refers to an antibody, a functional antibody fragment (e.g., Fab fragment) or a ligand such as an engineered binding protein that recognizes and binds an Fcα receptor on an effector cell. Preferred antibodies for use in the subject invention bind the Fcα receptor on an effector cell (white blood cell) at a site which is not bound by endogenous immunoglobulin A (IgA). Most preferably, the anti-Fcα receptor V_H and V_L portion binds a human FcαR. Preferred humanized anti-FcαR monoclonal antibodies are described, the teachings of which are fully incorporated herein by reference. The antibody that comprises the BsAb

or multispecific molecule of the invention may be whole, i.e. having heavy and light chains or any fragment thereof, e.g.. Fab or $(Fab')_2$ fragment. The antibody further may be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner *et al.* (U.S. Patent No. 4,946,778, issued August 7, 1990), the contents of which is expressly incorporated by reference.

An "effector cell" as used herein refers to an immune cell which is a leukocyte or a lymphocyte. Specific effector cells express specific Fc receptors and carry out specific immune functions. For example, monocytes, macrophages, neutrophils, eosinophils, basophils, and lymphocytes which express FcαR are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that present antigens. The expression of a particular FcR on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of FcγRI has been found to be up-regulated by interferon gamma (IFN-γ). This enhanced expression increases the cytotoxic activity of FcγRI-bearing cells against targets.

The recombinant antibodies or antibody fragments which specifically bind to an Fc receptor, are preferably "humanized" i.e. carry portions derived from a human antibody, but having at least a portion of a complementarity determining region (CDR) derived from a non-human antibody. Ordinarily that portion which is "humanized" is selected to provide specificity of the humanized antibody to bind a human Fc receptor. The humanized antibody has CDR portions derived from a non-human antibody and the "constant" portions of the antibody molecule are of human origin.

The portion of the non-human CDR inserted into the human antibody is selected to be sufficient for allowing binding of the humanized antibody to the Fcα receptor. A sufficient portion may be selected by inserting a portion of the CDR into the human antibody and testing the binding capacity of the created humanized antibody using flow cytometry or enzyme linked immunosorbent assay (ELISA).

All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to the Fc receptor.

An antibody can be humanized by any method, which is capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A,

filed on March 26, 1987), the contents of which is expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis as described in International Application WO 94/10332 entitled, *Humanized Antibodies to Fc Receptors for Immunoglobulin G on Human Mononuclear Phagocytes*.

In addition to an anti-Fcα receptor portion, the claimed multispecific molecules can comprise a binding determinant for a target i.e. an antibody, a functional antibody fragment or a ligand that recognizes and binds a pathogen (e.g., viruses, bacteria, fungi, protozoa), a pathogen infected cell, a cancer or tumor cell (e.g., breast, ovarian, prostate, testicular, lung, colon, rectum, pancreas, liver, central nervous system, head and neck, kidney, bone, blood, and lymphatic system) or other unwanted cell in a subject (e.g., a human or animal) or an antigen or modified form thereof. Additionally, the target portion may comprise or be directed against an antigen. A preferred embodiment contains an antigen that can be used to induce a specific immune response against a chronic infection, against a tumor or cancer cell, or to deplete antigen in the circulation. A different particularly preferred embodiment has an antigen that is attached to a multivalent molecule containing a binding determinant for an FcR, which stimulates the immune system by directing the antigen to an antigen presenting cell.

In one embodiment of the invention, the multispecific molecule contains a binding determinant or ligand which interacts with a molecule. In a preferred embodiment, the binding determinant binds a protein, e.g., a protein on a target cell, such as a cancer cell, or a cell of an infectious disease agent or the agent itself or an infected cell. Preferred binding determinants include antibodies, fragments of antibodies, and receptors for growth factors or differentiation factors. For example, a multivalent molecule can comprise an epidermal growth factor (EGF), or at least a portion or modified form that is capable of interacting with a receptor, e.g., an epidermal growth factor receptor EGF-R, or an antibody to EGF-R. A particularly preferred embodiment of the invention comprises a BsAb carrying a binding determinant for an human EGF-like receptor, including the EGF-R, HER2/*neu*, HER3, HER4, etc. In yet another preferred embodiment, the binding determinant is for the tumor antigen TAG 72 found e.g. on tumors of the breast, colon, and ovary.

In another preferred embodiment of the invention, the ligand is a small peptide, such as bombesin, gastrin-releasing peptide (GRP), litorin, neuromedin B, or neuromedin C. The sequences of the peptides can be found, e.g., in U.S. Patent No. 5,217,955, the content of which is incorporated herein by reference. The ligand can also be a modified form of any of these peptides. The modification can increase binding to

the receptor, decrease binding, or not affect the binding to a receptor. The modification of the ligand can also transform an agonist into an antagonist, such that the ligand inhibits rather than stimulates cell proliferation. Modification of the ligand can be an addition, a deletion, a substitution, or a modification of at least one amino acid.

In another preferred embodiment of the invention, a multispecific or bispecific molecule comprises an antigen. As used herein, the term "antigen" means any natural or synthetic immunogenic substance, a fragment or portion of an immunogenic substance, a peptidic epitope, or a hapten. In one embodiment of the invention, a bi- or multispecific molecule is employed to target an antigen, e.g., tetanus toxoid to the cell to enhance the processes of internalization and presentation by these cells, and ultimately, to stimulate an immune response therein. In a specific embodiment, the bispecific binding agent specifically binds the antigen (either directly, to an epitope of the antigen, or indirectly, to an epitope attached to the antigen) and, at the same time, binds a surface receptor of an antigen-presenting cell which can internalize antigen for processing and presentation. In another embodiment, the antigen is linked to the multi- or bispecific molecule and at the same time binds a surface receptor of an antigen-presenting cell. In a preferred embodiment the antigen is covalently attached to the multispecific molecule by genetic or chemical means The receptor-binding component of the bi- or multispecific molecule (and thus the bi- or multispecific molecule, itself) binds the receptor of the antigen-presenting cell at a site different and distinct from the naturally-occupying ligand. Thus, binding of the multispecific molecule occurs without competition by the natural ligand for the receptor. As a result, binding to the receptor will not be prevented by physiological levels of the ligand and the targeted receptor will remain capable of binding the molecule of the invention and the ligand.

One type of antigen can be an allergen. An "allergen" refers to a substance that can induce an allergic or asthmatic response in a susceptible subject. The number of allergens that elicit a sensitive response in a proportion of a population is enormous, and includes pollens, insect venoms, animal dander, dust mite proteins, fungal spores and drugs (e.g. penicillin). Examples of natural animal and plant allergens include proteins specific to the following genera: *Felis* (*Felis domesticus*); *Canis* (*Canis familiaris*); *Dermatophagoides (e.g. Dermatophagoides farinae*); *Periplaneta* (e.g. *Periplaneta americana)*; *Ambrosia* (*Ambrosia artemiisfolia*; *Lolium* (e.g. *Lolium perenne* or *Lolium multiflorum)*; *Cryptomeria* (*Cryptomeria japonica)* ; *Alternaria* (*Alternaria alternata)*; *Alder*; *Alnus* (*Alnus gultinosa)*; *Betula* (*Betula verrucosa)*; *Quercus* (*Quercus alba)*; *Olea (Olea europa)*; *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g. *Plantago lanceolata*); *Parietaria* (e.g. *Parietaria officinalis* or *Parietaria judaica)*;

Blattella (e.g. *Blattella germanica)*; *Apis* (e.g. *Apis multiflorum)*: *Cupressus* (e.g. *Cupressus sempervirens*. *Cupressus arizonica* and *Cupressus macrocarpa)*: *Juniperus* (e.g. *Juniperus sabinoides*. *Juniperus virginiana*. *Juniperus communis* and *Juniperus ashei*) *Thuya* (e.g. *Thuya orientalis*); *Chamaecyparis* (e.g. *Chamaecyparis obtusa*); *Agropyron* (e.g. *Agropyron repens)*; *Secale* (e.g. *Secale cereale)*; *Triticum* (e.g. *Triticum aestivum*); *Dactylis* (e.g. *Dactylis glomerata)*; *Festuca (e.g. Festuca elatior)*; *Poa* (e.g. *Poa pratensis* or *Poa compressa)*; *Avena* (e.g. *Avena sativa)*; *Holcus* (e.g. *Holcus lanatus)*; *Anthoxanthum (e.g. Anthoxanthum odoratum)*; *Arrhenatherum* (e.g. *Arrhenatherum elatius)*; *Agrostis* (e.g. *Agrostis alba)*; *Phleum (e.g. Phleum pratense)*; *Phalaris* (e.g. *Phalaris arundinacea)*; *Paspalum* (e.g. *Paspalum notatum*): *Sorghum* (e.g. *Sorghum halepensis*); and *Bromus* (e.g. *Bromus inermis)*.

Many allergens are found in airborne pollens of ragweed, grasses, or trees, or in fungi, animals, house dust, or foods. As a class, they are relatively resistant to proteolytic digestion. Preferable allergens are those which bind to IgE on mast cells and basophils, thereby causing a range of symptoms from inflammation and asthma to a type I anaphylaxis hypersensitivity reaction.

In another preferred embodiment, a binding determinant is specific for an antigen on an infectious disease agent or an infected cell, as defined *supra*. A binding determinant can be obtained from a polyclonal antibody, which can be produced by injection of a rabbit or other suitable animal with a fragment or component of a pathogen, for example, a cell wall component of a pathogen. IgG can be purified from the serum of the immunized animal, $F(ab')_2$ can be prepared, and used as the source of the target binding determinant. For example, polyclonal antibody prepared by immunizing a rabbit with a preparation of *Candida albicans* fragments can be obtained, and used to prepare anti-*C. albicans* IgG $F(ab')_2$, which can be coupled to a binding determinant for FcαRI by chemical methods described herein.

In some cases, it may be desirable to couple a substance which is weakly antigenic or nonantigenic in its own right (such as a hapten) to a carrier molecule, such as a large immunogenic protein (e.g., a bacterial toxin) for administration. In these instances, the bispecific binding reagent can be made to bind an epitope of the carrier to which the substance is coupled, rather than an epitope of the substance itself.

The antigen that can be linked either directly, or indirectly, to a multi- or bispecific molecule of the invention can be soluble or particulate; it may carry B cell epitopes, T cell epitopes or both. The antigen can be bacterial, fungal, viral or parasitic in origin. Often, the antigen will comprise a component of the surface structure of a pathogenic organism, or a surface structure in a cell infected by a pathogenic organism.

For example, the antigen can comprise a viral surface structure such as an envelope glycoprotein of human immunodeficiency virus (HIV) or the surface antigen of hepatitis virus. In addition, the antigen can be associated with a diseased cell, such as a tumor cell, against which an immune response may be raised for treatment of the disease. The antigen can comprise a tumor-specific or tumor-associated antigen, such as the HER-2/*neu* proto-oncogene product which is expressed on human breast and ovarian cancer cells (Slamon *et al.* (1989) *Science 244*:707). Another important cancer antigen which comprises a target of the BsAb of this invention is TAG 72, which has been identified on about 90% of colorectal cancers, 85% of breast tumors, and 95% of ovarian tumors (Johnson *et al.* 1986 *Cancer Res. 46*:850-897; Bodmer, M. *et al.*, European Patent Specification 0 348 442 B1; Mezes, P. *et al.* International Application WO 93/12231).

The cells of a subject can be exposed *ex vivo* or *in vivo* to the multispecific molecules of the invention, to target an antigen to antigen-presenting cells. Immune cells are separated and purified from subject blood, exposed to a multispecific molecule comprising the antigen, or the cells can be exposed to the antigen together with a multispecific molecule having a binding determinant for the antigen. After stimulation, cells are returned to the subject. Cells to be used in this procedure can also be treated with cytokines or other factors, for the purpose of, for example, up-regulating numbers of receptors per cell. Further, *in vivo* or *ex vivo* therapeutic use of the molecules can be enhanced by treatment of the subject with one or more cytokines or growth factors.

The method of this invention can be used to enhance or reinforce the immune response to an antigen. For example, the method is valuable for the treatment of chronic infections, such as hepatitis and AIDS, where the unaided immune system is unable to overcome the infection. It can also be used in the treatment of the acute stages of infection when reinforcement of immune response against the invading organism may be necessary.

The method can be used to reduce the dose of antigen required to obtain a protective or therapeutic immune response or in instances when the host does not respond or responds minimally to the antigen. Although generally desirable, the lowering of effective dose can be especially desirable when the antigen is toxic to the host such as is the case for allergies. Methods and uses for bi- or multispecific molecules comprising one or more antigens or comprising one or more binding determinants, e.g., an antibody interacting with an antigen, are further described in the published International Application WO 92/05793.

III. Methods for Making Multispecific Molecules

The multispecific molecules described above can be made by a number of methods. For example, both specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the multi-specific molecule is a mAb x mAb, mAb x Fab, Fab x $F(ab')_2$ or ligand x Fab fusion protein. A bispecific molecule of the invention can also be a single chain bispecific molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules. Methods for preparing bi- or multivalent antibodies are described for example described in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Binding of the single chain molecules to their specific targets can be confirmed by bispecific ELISA, familiar to those skilled in the art. Alternatively, each specificity of a multispecific molecule can be generated separately and the resulting proteins or peptides chemically conjugated to one another. For example, two humanized antibodies or antibody fragments can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains as described in Examples, *infra*.

The BsAbs of the present invention can be prepared by conjugating the anti-FcR and anti-target portions using methods described in the following Examples or those well-known in the art. For example, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (see e.g., Karpovsky *et al.* 1984 J. Exp. Med. 160:1686; Liu, MA *et al.*.1985 Proc. Natl. Acad. Sci. USA 82:8648). Other methods include those described by Paulus (Behring Ins. Mitt. 1985 No. 78, 118-132); Brennan *et al.* (Science 1985 229:81-83), and Glennie *et al.* (J. Immunol. 1987 139: 2367-2375). Examples of other cross-linking agents include ortho-phylenedimaleimide (o-PDM), protein A, carbodiimide. In the preferred embodiment for BsAb, the conjugating agent is o-PDM. Other preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

Based on their ability to bind FcR bearing immune cells and specific target cells. a particular embodiment of a multispecific molecule can be administered to a subject to treat or prevent reoccurrence of a variety of diseases or conditions, including: cancer (e.g., breast, ovarian, testicular, prostate, lung, brain, colon, rectum, pancreas, liver, central nervous system, head and neck, kidney, bone, blood and lymphatic system), pathogenic infections such as viral (such as HIV, HTLV and FELV), protozoan (such as *Toxoplasma gondii*), fungal (such as *Candida albicans*); and bacterial (such as *Staphylococcus aureus*, *Streptococcus hemolyticus* and *Mycobacterium tuberculosis*). Another aspect of the invention provides molecules that are useful for vaccination against diseases and cancer by including an antigen from disease organisms, from infected cells, from gene products of disease organisms or from cancer cells. For these purposes, the invention provides compositions which are multispecific molecules that link the useful operative antigen to a binding determinant that directs the antigen to the immune system. An Example provided herein describes a molecule which functions to target tetanus toxoid directly to FcαR on monocytes, resulting in stimulation of T cells at lower doses than is required by free tetanus toxoid.

For use in therapy, an effective amount of an appropriate multispecific molecule can be administered to a subject by any mode that allows the molecules to exert their intended therapeutic effect. Preferred routes of administration include oral, transdermal (e.g., via a patch), and injection (subcutaneous, intravenous, parenteral, intraperitoneal, intrathecal, etc.). The injection can be in a bolus or a continuous infusion.

Further, the cells of a tissue, e.g. blood, may be removed from a patient, fractionated and cultured if appropriate to expand the cell number, treated *ex vivo* with the multispecific multivalent composition in a pharmaceutically acceptable carrier, and returned to the patent for therapy. During the *ex vivo* culture and expansion, a particular cell type may be selected, e.g. a monocyte population. Further, *ex vivo* cultured cells may be treated at various points during *ex vivo* culture and expansion, with agents to modify certain functional FcαR molecules. Agents include but are not limited to, growth factors, cytokines, lymphokines such as IFN-γ, G-CSF, TNF, and GM-CSF, and interleukins such as IL-2, IL-10 and IL-12.

A multispecific molecule is administered in conjunction with a pharmaceutically acceptable carrier. As used herein, the phrase "pharmaceutically acceptable carrier" is intended to include substances that can be coadministered with a multispecific molecule and allows the molecule to perform its intended function. Examples of such carriers include solutions, solvents, dispersion media, delay agents,

emulsions and the like. The use of such media for pharmaceutically active substances are well known in the art, and are discussed *infra*. Any other conventional carrier suitable for use with the molecules falls within the scope of the instant invention. Further, therapy with the multispecific multivalent binding molecule may be coordinated into a treatment regimen with other similar molecules, or with traditional chemotherapeutic agents such as cis-platin, AZT, DDI, adriamycin, tetracycline, cefachlor, nystatin, and acyclovir.

Combinatorial libraries can be screened to obtain members of the library with a desired binding activity, and to identify the active species, by methods that have been described (see, e.g., Gordon *et al.*, *J Med. Chem.*, *op. cit.*). These include affinity chromatography with an appropriate "receptor" to isolate ligands for the receptor, followed by identification of the isolated ligands by conventional techniques (e.g., mass spectrometry and NMR). Preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, spectrophotometric enzymes, radioisotopes, or luminescent compounds) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor.

Combinatorial libraries of compounds can also be synthesized with "tags" to encode the identity of each member of the library (see, e.g., W.C. Still *et al.*, International Application WO 94/08051). In general, this method features the use of inert but readily detectable tags, that are attached to the solid support or to the compounds. When an active compound is detected the identity of the compound is determined by identification of the unique accompanying tag. This tagging method permits the synthesis of large libraries of compounds which can be identified at very low levels among to total set of all compounds in the library.

Specific binding proteins with high affinities for targets can be made according to methods known to those in the art. For example, proteins that bind specific DNA sequences may be engineered, and proteins that bind a variety of targets, especially protein targets (Ladner, R.C., *et al.*, U.S. Patent 5,233,409; Ladner, R.C., *et al.*, U.S. Patent 5,403,484) may be engineered and used in the present invention as the FcαR binding determinant or as the target binding determinant, as appropriate. Further, the methods of these libraries can be used in screens to obtain binding determinants that are mimetics of the structural determinants of antibodies.

In particular, the Fv binding surface of a particular antibody molecule interacts with its target ligand according to principles of protein-protein interactions, hence sequence data for V_H and V_L (the latter of which may be of the κ or λ chain type)

is the basis for protein engineering techniques known to those with skill in the art. Details of the protein surface that comprises the binding determinants can be obtained from antibody sequence information, by a modeling procedure using previously determined three-dimensional structures from other antibodies obtained from NMR studies or crytallographic data. See for example Bajorath, J. and S. Sheriff, 1996, *Proteins: Struct., Funct., and Genet. 24 (2)*, 152-157; Webster, D.M. and A. R. Rees, 1995, "Molecular modeling of antibody-combining sites," in S. Paul, Ed., *Methods in Molecular Biol. 51*, Antibody Engineering Protocols, Humana Press, Totowa, NJ, pp 17-49; and Johnson, G., Wu, T.T. and E.A. Kabat, 1995, "Seqhunt: A program to screen aligned nucleotide and amino acid sequences," in *Methods in Molecular Biol.51*, *op. cit.*, pp 1-15.

Breast and ovarian cancers are sex hormone dependent cancers. Breast tumors may be characterized by abnormally expressed receptors, e.g. those of the human-EGF-like receptor family (HER), for example HER-2, -3, and 4. The invention is not limited to these embodiments of HER antigens. The natural HER ligand, Heregulin, can be incorporated into a bispecific antibody (BsAb) or multispecific molecule, as a means to target a breast tumor cell expressing one or more HER receptor during cancer. Further, a heregulin molecules are binding determinants for heterodimeric HER receptors containing, eg. a monomer of each of HER-2, -3 or -4 in combination.

Additional examples of sex hormone-dependent cancer include prostate cancer (Smith, P. (1995), *Cancer Surveys Vol. 23: Preventing Prostate Cancer*, Imper. Cancer Research Fund and testicular cancers). The growth of hormone-dependent cancer types is promoted by male hormones (e.g., androgens such as testosterone and dihydrotestosterone). Removal of the testes (castration) was for many years the standard method of preventing secretion of male hormones by the gonads, to reduce growth of the cancer. Currently, secretion of male hormones is suppressed by chemical means by interfering with production of luteinizing hormone (LH), which regulates synthesis of male hormones. Similar considerations are applicable to other sex hormone-dependent cancers, such as breast or ovarian cancer, so that patients with these diseases or in a population prone to these diseases, are usually not administered sex hormones as therapeutic or replacements. Multispecific molecules of the invention can comprise binding determinants for sex hormones, to reduce the concentration and suppress tumor growth.

In a preferred embodiment, the methods of this invention include administration, for example, to a cancer patient, of a multispecific multivalent binding

molecule preparation comprising at least one binding determinant with affinity for a tumor marker or a tumor-specific protein of the cancer to be treated. for example, the nestin protein for brain cancers. The nestin protein. which is expressed during normal mammalian fetal development, is also expressed on tumors of the central nervous system, including most forms of brain cancer (McKay, D.G. Ronald, *U.S. Patent No. 5,338,839*. 8/16/94). It is also expressed on melanomas on the skin and on melanomas that have metastasized (V.A. Florenes, R. Holm. O. Myklebost, U. Lendahl, O. Fodstad. *Cancer Res. 54*: 354-6, 1994), to other organs and are difficult to detect and treat. The preferred site of delivery for treatment of a brain tumor with the molecules of this invention is directly into the central nervous system or directly, to the brain via spinal injection or fine needle delivery. For a metastatic cancer, a preferred delivery route would be by direct injection into the circulation. or by the *ex vivo* blood methods described herein.

Other tumor types for which the methods of this invention are exemplified by, but are not limited to, Wilm's tumor (A.J. Buckler. *et al.* U.S. Patent No. 5,350,840) a pediatric kidney cancer due to occurrence of a somatic mutation in the patient's single copy of a gene normally found in two intact copies. Wilm's tumor can be cured surgically in 95% of cases, and a bispecific or multispecific multivalent binding protein is envisioned to be suitable as an adjunct therapeutic modality for surgical patients. Other examples of known cancer-associated proteins for which the compositions of matter and methods of the current invention are suitable include those associated with gastrointestinal cancer (R. Fishel *et al.*, International Application WO 95/14085, 05/26/95), those characterized by development of multiple drug resistance during chemotherapy (J.M. Croop *et al.*, U.S. Patent No.5,198,344), and a large number of oncogenes well known to the skilled artisan such as *Rb*, *ras*, and *c-myc*, the sequences of which are available for analysis to those with skill in the art. The compositions of this invention are, for example, suitable for inhibition of secreted enzymes such as matrix metalloproteinases, which are considered to potentiate tumor metastasis (Liotta, L.A., *et al.* 1991 *Cell* 64:327-336). In the latter embodiment, a multispecific binding molecule with a binding determinant to the matrix metalloproteinase and another for FcαR would facilitate inhibition and clearance of these enzymes from *in situ* activity. If used in conjunction with standard surgical and chemotherapeutic regimens, the compositions are foreseen to reduce cancer re-occurrence and enhance long-term survival.

IV. Pharmaceutical Compositions

The compounds of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject *in vivo*. In a preferred embodiment, the pharmaceutical composition comprises either a multispecific molecule (compound, or agent) of the invention and a pharmaceutically acceptable carrier. In yet another embodiment of the present invention, the pharmaceutical composition can be administered by combination therapy, i.e., combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one anti-cancer agent, at least one antibiotic, at least one cytokine, at least one vaccine, or other conventional therapy. Exemplary anti-cancer agents include cis-platin, adriamycin, and taxol. Exemplary antibiotics include isoniazid, rifamycin, and tetracycline. Exemplary cytokines include G-CSF, GM-CSF, interleukins and interferons.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound, or for controlled release.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and di-carboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route

and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol.* 7:27). Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient

which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent.

Transdermal patches offer the advantage of providing controlled delivery of a compound of the present therapeutic inventions to the body. Such dosage forms can be made by dissolving or dispersing the composition in the proper medium. Absorption enhancers can also be used to increase the flux of the composition across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the composition in a polymer matrix or gel. Devices, including patches, which transdermally deliver a composition by iontophoresis or other electrically-assisted methods can also be employed in the present invention, including, for example, the devices described in U.S. Patent Nos. 4,708,716 and 5,372,579.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, *supra*, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.01 to 99.5% (more preferably, 0.1 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a compositions of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic compositions may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4.,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for

continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

In certain embodiments, the compounds of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs , thus enhance targeted drug delivery (see, e.g., V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, e.g., U.S. Patent 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor or infection. The composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

A "therapeutically effective dosage" preferably inhibits tumor growth or pathogen infection by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit cancer or infectious disease can be evaluated in an animal model system predictive of efficacy in human tumors and infectious diseases. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition in vitro by assays well-known to the skilled practitioner. A therapeutically effective amount of a

therapeutic compound can decrease tumor size, prevent or delay death of infected tissues or organs, decrease fever and while cell count, improve CD4 count or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application, are hereby expressly incorporated by reference.

EXAMPLES

The following methodology described in the Materials and Methods section was used throughout these Examples, set forth below.

Materials and Methods

Cell lines and Monoclonal antibodies

The murine hybridoma producing cell line for the anti-FcαR antibody used to construct the BsAb compositions described herein is A77 (Monteiro *et al.* 1992, J. Immunol. 148: 1764-1770), and other anti-FcαRI hybridoma cell lines with properties the same or similar to A77, such as A3, A59 and A62 ((Monteiro *et al.*, 1992), can be used also. The hybridoma cell line for the anti-HER2/*neu* antibody is 520C9 (Frankel, A. *et al.* 1985 *J. Biol. Response Modifiers* 4:273-286; available from American Type Culture Collection, ATCC, 12301 Parklawn Drive, Rockville, MD 20852; accession number HB8696). Antibody H425 is a humanized anti-EGF-R antibody (Kettlesborough, C. *et al.* , 1991, Prot. Eng. 4: 773-783). CC49 (anti-TAG 72) hybridoma was obtained from the ATCC (accession number HB 9459). mAb-producing cell lines were cultivated in Iscove's Modified Dulbecco's Medium (IMDM, Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). The mAb preparations were purified from the respective hybridoma supernatants by protein A affinity chromatography (Bio-Rad, Richmond, CA).

SKBR-3 (Backman *et al.* Cancer Res 54: 2456-2461), a human breast carcinoma cell line, and SKOV-3 (ovarian carcinoma), which overexpress the HER2/*neu* protooncogene, were obtained from ATCC. EGF-R over-expressing cell lines A431 (skin carcinoma), HN5 (head and neck carcinoma) and MDA-MB-468 (breast

carcinoma), were obtained from ATCC. Tumor cell lines were cultivated in Iscove's Modified Dulbecco's Medium (IMDM, Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). The monocytoid cell line U937 (J. Immunol. 136: 1641-1647, 1986) which expresses FcαR was obtained from the ATCC and grown in RPMI-1640 plus 10% FBS (Gibco/BRL, Grand Island, NY).

Candida albicans (strain ATCC 448,585) was grown in Sabouraud maltose broth (Difco, Detroit, MI).

Preparation of Blood Cells

Leukocytes were prepared from heparinized whole venous blood or from apheresis of normal human volunteers. Whole blood was diluted with RPMI containing 5% dextran at a ratio of 2.5:1 (v/v). The erythrocytes were allowed to sediment for 45 minutes on ice, then the cells in the supernatant were transferred to a new tube and pelleted by centrifugation. The residual erythrocytes were removed by hypotonic lysis. The remaining lymphocytes, monocytes and neutrophils were kept on ice until use in binding assays. For some experiments, neutrophils were separated from mononuclear cells by ficoll hypaque (Pharmacia-Upjohn, Piscataway, NJ) gradient separation. Monocytes were enriched from mononuclear cells by cold aggregation and settling through a cushion of fetal calf serum. Monocyte cultures were used fresh or were incubated at 37°C, 5% CO_2 for 24 to 48 hours in teflon dishes at 4 x 10^6 cells/ml of MSFM containing 2.0% normal human serum type AB (Sigma, St. Louis, MO) and 500 IRU/ml IFN-γ (R&D Systems, Minneapolis, MN). Neutrophils were cultured for 24 to 48 hours (37°C, 5% CO_2) in AIM V media (Gibco/BRL, Grand Island, NY) with 50 ng/ml G-CSF (Kindly provided by R. Repp, U. of Erlanger, Germany) and 500 IRU/ml IFN-γ.

Binding by flow cytometry

The binding of the BsAb to FcαR and HER2/*neu* was assessed by flow cytometry. Various concentrations of BsAb diluted in PBS, pH 7.4 containing 2mg/ml BSA and 0.05% NaN_3 (PBA), were incubated with SKBR-3 cells or with human leukocytes for one hour at 0°C. The cells were washed with PBA and incubated with a phycoerythrin labeled goat anti-mouse antibody for one hour at 0°C. The cells were washed and fixed with 1% paraformaldehyde, and cell associated fluorescence was analyzed on a Becton Dickinson (Mountain View, CA) FACScan.

To assess whether IgA binding interfered with mAb A77 binding, A77 was incubated with TNF-treated cells in the presence of an excess of human IgA, and

compared to controls incubated in the absence of IgA. A77 binding was detected with a phycoerythrin-labeled goat anti-mouse antibody as above. Cells were washed and fixed with 1% paraformaldehyde, and cell associated fluorescence was analyzed on a Becton Dickinson FACScan. In addition, binding human IgA to U937 cells was assessed in the presence of A77 mAb, and compared to controls in the absence of excess A77 mAb. IgA binding was detected with FITC labeled anti-human IgA antibody

BsAb coupling procedure

BsAb preparations were constructed using the method of Glennie *et al.* (J. Immunol. 1987 139: 2367-2375). mAbs A77 (anti-FcαR), 520C9 (anti-HER2/*neu*), CC49 (anti-TAG 72) and H425 (anti-EGF-R) antibodies were produced by *in vitro* cultivation of the respective hybridoma cell lines. The antibody preparations were each digested with pepsin to produce $F(ab')_2$ preparations, and subsequently reduced to Fab' by addition of 10 mM mercaptoethanolamine (MEA) and incubation for 30 minutes at 30°C. The Fab' fragments were applied to a Sephadex® G-25 (Pharmacia-Upjohn, Piscataway, NJ) column equilibrated in 50mM Na Acetate, 0.5mM EDTA, pH 5.3 (4°C). One-half volume of ortho-phenylenedimaleimide (o-PDM, 12mM) dissolved in dimethylformamide and chilled in a methanol/ice bath was added to the 520C9 Fab', and the mixture was incubated 30 minutes at 0°C. The Fab'-maleimide was then separated from free o-PDM on Sephadex G-25 equilibrated in 50mM Na Acetate, 0.5mM EDTA, pH 5.3 (4°C).

For preparation of BsAb, the 520C9 Fab'-maleimide was added to an equimolar solution of the A77 Fab'. The reactants were concentrated under nitrogen to the starting volume using a Diaflo® membrane in an Amicon® (Lexington, MA) chamber, at 4°C, for 18 hours. The pH was then adjusted to 8.0 with 1M Tris-HCl, pH 8.0, and the mixture was reduced with 10mM MEA (30 minutes, 30°C) and alkylated with 25 mM iodoacetamide. A77XCC49 and A77XH425 were produced using similar procedures. The bispecific $F(ab')_2$ reactant preparation was separated from unreacted Fab's and other materials using a Superdex® 200 column (Pharmacia-Upjohn, Piscataway, NJ) equilibrated in PBS.

Analysis of A77X520C9 BsAb by HPLC showed that this BsAb comprised of two main species; 75-85% of $F(ab')_2$ heterodimer (100kDa, 10.43 mins) and 15-25% of $F(ab')_3$ heterotrimer (~150 kDa, 9.86 mins) (Figure 1). Based on the method of preparation, the $F(ab')_3$ species is expected to be comprised of two 520C9 F(ab') and one A77 F(ab'). A control experiment, in which a F(ab')-o-PDM derivative was incubated without a non-derivatized second F(ab'), confirmed that F(ab')-o-PDM

derivative does not cross-link with itself since all the hinge-SH groups are occupied by o-PDM and no free -SH group is available for linkage (data not shown). Thus the $F(ab')_2$ and $F(ab')_3$ species in these BsAb preparations are hetero-complexes of F(ab') fragments with two different specificities.

Antibody dependent cellular cytotoxicity (ADCC)

Human breast carcinoma cells, SKBR-3, which over-express HER2/*neu*, were used as target cells for determination of lysis using multispecific compositions comprising binding determinants for HER2/*neu*. Other target cell lines were used in tests of molecules with different antigen-binding determinants, for example, A431 cells for EGF-R, KLEB for TAG 72, *etc*. Effector cell samples were obtained by using heparinized whole blood, purified neutrophils (purified as described *supra*), or monocytes prepared from Leukopaks® obtained from Advanced Biotechnologies Inc. (Columbia, MD) as previously described (Guyre, P.M. *et al.* 1989, *J. Immunol. 193*: 1650). To prepare for use as effector cells, monocytes were cultured in Teflon® containers in Macrophage Serum-Free Medium (Gibco/BRL) containing 2% human serum for 24 to 48 hours. Target cells were labeled with 100 μCi of ^{51}Cr for one hour prior to combining with effector cells and antibodies in a U-bottom microtiter plate. After incubation for 16 to 18 hours at 37°C, supernatants from each well were collected and analyzed for radioactivity. Cytotoxicity was calculated by the formula: % lysis = (experimental CPM - target leak CPM/detergent lysis CPM - target leak CPM) X 100%. Further, specific lysis = % lysis with antibody - % lysis without antibody. Assays were performed in triplicate.

Fluoresceination of Antibodies

The pH of mAb solution was adjusted to 9.3 by the addition of 0.1M Na_2CO_3. Fluorescein iso-thiocyanate (FITC, Sigma, St. Louis, MO) was dissolved in DMSO at a concentration of 2mg/ml. Forty μg of FITC was added for each milligram of mAb and incubated for two hours at room temperature. The fluoresceinated mAb was separated from the free FITC by G-25 chromatography.

Modulation of FcαR by mAb A77

The ability of mAb A77 to modulate the number of FcαR on the surface of human monocytes was assessed by incubating monocytes with various dilutions of MAb A77 37°C for 18 hours (or with mAb 520C9 as an isotype control). Monocytes were then washed with PBA, and incubated for one hour at 0°C in the presence of

human serum IgA at 100μg/ml. Cells were further washed with PBA, and IgA binding to FcαR, was detected with FITC-labeled anti-human IgA antibody. Percent modulation = 1 - (MFI of the sample/MFI of the control) x 100%, where MFI is the mean fluorescence intensity.

BsAb-mediated Phagocytosis

Assay of monocyte and neutrophil-mediated phagocytosis of SKBR-3 cells was performed with SKBR-3 target cells labeled with the lipophilic red fluorescent dye PKH 26. Buffy coat cells purified from heparinized whole blood containing monocytes, neutrophils, and lymphocytes were incubated with the labeled targets at 37° for 6 hours in the absence or presence of BsAb. Monocytes and neutrophils were stained with FITC labeled anti-CD14 mAb (AML-2-23) at 0°C, and cells were washed and analyzed by two color fluorescence by FACScan. Percent phagocytosis is expressed as the percent of effector cells (monocytes or neutrophils) that have PKH 26 stain associated with them.

Example 1: Bispecific Antibody A77X520C9 Binds Effector Cells

To determine efficacy of the bispecific antibody BsAb A77X520C9 in killing breast cancer cells in a patient, ability to specifically bind to breast cancer cells in culture was determined. For these experiments, cells of line SKBR-3, which overexpresses the HER2/*neu* oncogene, were used. The 520C9 binding determinant derives from an anti-HER2/*neu* murine hybridoma (Frankel, A. *et al.*, 1985 J. Biol. Response Modifiers 4: 273-286).

Figure 2 shows that BsAb A77X520C9 bound to each of two types of effector cells, neutrophils (PMNs) and monocytes. Figure 3 shows that, in whole blood, A77X520C9 BsAb binds to FcαRI-expressing PMN and monocytes, but not to FcαR-negative lymphocytes. This binding activity was not inhibited by the physiological levels of serum IgA in whole blood, which is sufficient to saturate all the FcαRI expressed *in vivo*.

Example 2: Bispecific Antibody A77X520C9 Binds Target Breast Cancer Cells

The BsAb A77X520C9 bound to the target breast tumor cells SKBR-3 derived from a breast tumor (Fig. 4) to the same extent as did the previously described BsAb MBX210 (Valone *et al.* 1995 J. Clin. Oncol. 13(9): 2281-2292). Mean fluorescence intensity (MFI) as a measure of binding was found to increase as a function of BsAb concentration when breast tumor cells were incubated with each of 0.1, 1.0 or

10 μg/ml. The fraction of A77X520C9 BsAb that bound to breast tumor target cells was equivalent to or greater than the fraction of the control BsAb MDX210 (Fig. 4) and binding to effector cells (Fig. 2) was similar as a function of concentration of each of the BsAbs.

Further data showing binding of A77X520C9 BsAb to HER2/*neu* positive SKBR-3 cells is shown in Figure 5. As expected, only the BsAb and 520C9 $F(ab')_2$, but not A77 $F(ab')_2$ control, bound the target breast tumor cells.Data are shown in Figures 7-12 with a variety of effector cells, with several independent bispecific antibody preparations, and with varying concentrations of different preparations of BsAb.

Example 3: <u>Bispecific Antibody A77XH425 Binds Target Skin Carcinoma Cells</u>

The monoclonal antibody H425 is specific for the EGF receptor (EGF-R), which is overexpressed on cell lines derived from skin carcinoma (A431), and MDA-MB468 (breast carcinoma). The bispecific molecule A77XH425 which carries a binding determinant for EGF-R and for FcαRI, was prepared by the same chemical coupling procedure as A77X520C9, and tested for binding to target cells by the methods described herein.

Similar to binding of A77X520C9 to HER2/*neu*-bearing target cells shown in Example 2 and Figures 2-5, Figure 6 shows that the EGF-R tumor-specificity of the BsAb A77XH425 causes this molecule to be bound to A431 tumor cells which overexpress EGF-R. Further, the tumor binding determinant H425 $F(ab')_2$, but not A77 $F(ab')_2$, which bears the FcαRI determinant, bound to the A431 target cells which overexpress EGF-R.

Example 4: <u>Binding of Bispecific Antibody A77X520C9 Mediates Effector Leukocyte Cytolysis of HER2/*neu*-bearing Target Cells</u>

Following determination of the ability of A77X520C9 to bind to breast tumor cells, its ability to bind both to target cells and to specific leukocytes such that cytolysis of the breast tumor cells is effected was determined also. Thus, antibody-dependent cellular cytolysis (ADCC) mediated by bispecific antibodies (BsAb; also referred to as bispecific molecules, BSM) of the invention is shown in Figures 7-16 as percent cytotoxicity or percent lysis.

As shown in Figure 7, cytotoxicity of SKBR-3 cells mediated by A77X520C9 and neutrophil effector cellss (PMN) varied from 6% to 9% at 0.1 μg/ml, and increased to 20% to 30% at 1.0 μg/ml. Similar data are shown for killing mediated by effector cells in whole blood in Figure 8. In Figure 9 this cytotocity mediation by

BsAb is shown to be inhibited by addition also of the FcαRI-binding A77 F(ab')$_2$. but not that of FcγRI determinant of M22 F(ab')$_2$.

Therapy of subjects with multispecific multivalent chemical compositions directly administered into the circulation requires that these agents function in whole blood. Ability to function in whole blood, i.e., to mediate cytolysis, is shown in Figure 8, in which A77X520C9 preparations were found to mediate cytolysis of cultured breast tumor cells by blood effector cells. At 0.1 μg/ml, between 15% and 20% of the tumor cells were lysed by A77X520C9, and at 1.0 μg/ml cytolysis was approximately 25% to 30% of breast tumor cells. Since whole blood contains IgA at a concentration of 2 to 5 mg/ml, these data also show that cytotoxic activity of this BsAb is not inhibited by IgA. These results show that BsAb can be delivered for therapeutic application *in vivo*.

BsAb-mediated destruction of tumor cells by FcαRI-expressing cytotoxic effector cells was examined using freshly purified effector cells (monocytes and PMN) as well as whole blood as the source of effector cells. Figure 10 shows that 1.0 μg/ml of A77X520C9 BsAb mediated up to 37% BsAb-dependent lysis of HER2/*neu* positive SKBR-3 cells by purified PMN (neutrophils). This cytotoxic activity was dose dependent and saturated at 1.0 μg/ml of the BsAb. Figure 11 shows that A77X520C9 mediated up to 40% BsAb-dependent lysis of SKBR-3 cells when purified monocytes were used as effector cells. Finally, A77X520C9 BsAb mediated up to 40% BsAb-dependent lysis of the same target cells when whole blood was used as a source of effector cells (Figure 12). In the data shown in Figures 10-12, A77 F(ab')$_2$ with the determinant for FcαRI, inhibited the ADCC activity of this BsAb, but the anti-CD64 M22 F(ab')$_2$ with the determinant for FcγRI, d id not. The background lysis (in the absence of BsAb) in these experiments was about 10%.

Similarly, A77X520C9 BsAb mediated lysis of another HER2/*neu* over-expressing tumor line, SKOV-3 (ovarian carcinoma line), in a whole blood ADCC assay.

Example 5: Binding of Bispecific Antibody A77X5H425 Mediates Effector Leukocyte Cytolysis of EGF-R-bearing Target Cells

Similar to the data shown for BsAb for the tumor antigen HER2/*neu*, A77XH425 BsAb, with the affinity determinant for EGF-R, mediated up to 52% BsAb-dependent lysis of A431 target tumor cells with purified PMN as effector cells (Figure 13), up to 55% BsAb-dependent lysis with purified monocytes as effector cells (Figure 14), and up to 43% BsAb-dependent lysis with the whole blood a source of effector cells (Figure 15). In these experiments A77 F(ab')$_2$, but not M22 F(ab')$_2$, inhibited the cytotoxic activity. The background lysis (without BsAb) was about 10%.

These results show that the BsAb A77X520C9 can bind both effector and HER2/*neu*-bearing target cells, and can mediate target cytolysis by purified neutrophils and monocytes, and by these effector cells in whole blood. Lymphocytes in whole blood did not bind the BsAb. Further, the BsAb A77XH425 can bind target cells that overexpress EGF-R, and mediated cytolysis of these cells in the presence of effector cells.

In whole blood ADCC assays, A77XH425 BsAb-dependent lysis (40-50% cytolysis) of HN5 (head and neck carcinoma line) and MDA-MB468 (breast carcinoma line), which express comparable levels of EGF-R as A431 (skin carcinoma line), was observed.

Example 6: Bispecific Antibody anti-TAG 72XA77 Mediates Cytolysis of TAG 72-Bearing Tumor Cells

The Examples above show that BsAb preparations are effective in mediating cytolysis of cell of cell lines derived from tumors, bearing the tumor antigens HER2/*neu*, and EGF-R. To extend the potential use of BsAb, another composition with a binding determinant for the TAG 72 tumor marker was prepared by the coupling method described *supra*. The mucine antigen TAG 72 is found in the majority of breast, colon, ovarian and other cancers. A variety of antibodies that specifically bind TAG 72 are available, for example the monoclonal antibody produced by hybridoma CC49 (ATCC HB 9459, Mezes, P. *et al.*, International Application WO 90/04410).

CC49 was coupled to A77 to produce BsAb anti-TAG 72XA77, to target tumor cells bearing the TAG 72 antigen specifically to effector cells bearing FcαR. Figure 16 shows neutrophil mediated antibody-dependent cytotoxicity by the BsAb anti-TAG 72XA77 (constructed from CC49 and A77 mAb antibodies) of TAG 72-bearing tumor cells, as a function of concentration in μg/ml. The results in Figure 16 show that BsAb anti-TAG 72XA77 mediated cytolysis of tumor cells to a similar extent as BsAbs A77X520C9 for cells bearing HER2/*neu*, and A77XH425 for cells bearing EGF-R, shown in the Figures above.

Overall, Examples 1-6 demonstrate cytolysis of tumor cells bearing three different tumor antigens mediated by the bispecific molecules of the invention. These Examples and other studies performed using cell lines taken from breast tumors, skin carcinoma, gastric carcinoma, head and neck carcinoma, and ovarian carcinoma, show that FcαRI-directed BsAb preparations coupled to binding determinants for a variety of tumor antigens can be broadly applicable in a variety of malignancies. This observation is significant because target antigen restriction of FcγR-mediated ADCC activity of

PMN has been reported for B cell lymphoma associated antigens (Elässer, D., *et al.*, 1996. *Blood.* 87,9:3803-3812).

Example 7: BsAb Cytolysis Requires Access to FcαR Via the A77 Binding Determinant

To show that BsAb-mediated cytolysis of target cells is due to FcαR recognition by the A77 binding determinant, BsAb cytolysis was analyzed in the presence of A77 $F(ab')_2$ in Figures 9-15. If BsAb-mediated cytolysis functions by binding to FcαR because of the A77-derived binding determinant, then the addition of A77 $F(ab')_2$, but not an antibody with a different receptor binding determinant, could cause inhibition of cytolysis of breast tumor cells.

The results shown in Figures 9-15 show that BsAb-mediated cytolysis functions by binding to FcαRI via the A77-derived binding determinant. In Figures 9-12, the addition of A77 $F(ab')_2$ to the mixture of A77X520C9, target cells, and effector cells causes inhibition of cytolysis, while similar addition of an antibody that does not share the FcαRI has no effect on level of cytolysis. Figures 13-15 show this result also with BsAb A77XH425-mediated cytolysis of tumor cells: inhibition was observed in the presence 50 μg/ml A77 $F(ab')_2$, and not in the presence of 50 μg/ml of M22 $F(ab')_2$. M22 $F(ab')_2$ specifically binds a different receptor, the FcγRI receptor (Valone *et al.*, 1995 *J. Clin. Oncol. 13*: 2281-2292).

These data show that BsAbs A77X520C9 and A77XH425 mediate cytolysis of tumor cells in a manner that is dependent on specific binding of the BsAb to FcαRI on effector cells. Further, inhibition of BsAb killing by A77 $F(ab')_2$ was found regardless of the nature of the effector cells.

Example 8: Stimulation of T-cell Growth by Antigen-Presentation Using a Bispecific Antibody

For use of a BsAb for delivery of an antigen, the following procedure was used to couple the FcαRI binding determinant of mAb A77 to tetanus toxoid. Purified tetanus toxoid (TT, Accurate Chemical and Scientific Company, Westbury, NY) was reacted with sulfo-succidimidyl, 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (s-SMCC, Pierce, Rockford, IL) at a molar ratio of 20 SMMC: 1 TT for two hours at room temperature. Free SMCC was removed from TT:SMCC by G-25 chromatography. The F(ab')2 fragment of mAb, A77, was reduced to Fab' by incubating in the presence of 5 mM mercaptoethylamine (MEA, Sigma, St. Louis, MO) for 30 minutes at 30°C. Free MEA was removed from Fab' by G-25 chromatography. The A77 Fab' was incubated with the SMCC-treated TT for two hours at room temperature followed by an overnight

incubation at 4°C. The A77-TT conjugate was purified from uncoupled Fab' by Superdex® 200 gel filtration (Pharmacia-Upjohn, Piscataway, NJ).

To assay T cell proliferation following antigen presention. TT-specific T cell lines were generated from immunized individuals as previously described (Gosselin, E.J. *et al. J. Immunol.* 179:3477, 1992), monocytes were purified by cold aggregation as described previously (Guyre, P.M. *et al. J. Immunol.* 143:1650, 1989) and T cells (50μl of 10^6/ml) and autologous monocytes (50μl of $5X10^5$/ml) were added to wells of a 96 well tissue culture plate in the presence of various concentrations of TT or of A77-TT and incubated at 37° in a CO_2 incubator. The relative number of T cells in each well was assessed after incubation for four days by measuring lactate dehydrogenase (LDH) released from cells after lysis using a kit purchased from Promega (Madison, WI). LDH levels were quantified spectrophotometrically after addition of substrate and stop solution. Optical density was read at 490 nm using an ELISA plate reader (Molecular Devices, Palo Alto, CA).

Each data point presented in Figure 17 is the mean of data obtained from quadruplicate samples, with background value (monocytes and T cells in the presence of media only) subtracted. The A77-TT conjugate induced equivalent T cell proliferation at antigen doses that were 30-100 fold lower than that of the uncoupled TT. These data show that directing the TT antigen to FcαRI by coupling to mAb A77 clearly enhanced monocyte presentation of TT to TT-specific T cells. Similar antigen presentation can be achieved with antigens for allergies, such as antigens of the dust mite, and cat and dog antigens, and for vaccine antigen presentation, such as for malaria, chicken pox, hepatitis virus C, and other non-A-non-B hepatitis viruses.

Example 9: Bispecific Antibody Binds Target Cells and Causes Phagocytosis by Effector Cells

To demonstrate BsAb mediation of phagocytosis, the following experiments were performed to determine the extent to which effector cells engulf SKBR-3 breast tumor cells or cell fragments in the presence of BsAb A77X520C9. These studies tested phagocytosis of SKBR-3 by neutrophils, monocytes, and monocyte-derived macrophages (MDM). Phagocytosis of tumor cells mediated by FcαRI has not been described.

Tumor cells were labeled prior to exposure to leukocytes with lipophilic red fluorescent dye PKH26. Following incubation of effector cells with target cells for 6 hours, effector monocytes and neutrophils purified from whole blood were stained with FITC-labeled anti-CD14 mAb, and were analyzed by cell sorting. The results are shown in Figures 18-20. Percent phagocytosis is expressed as FITC-stained effector cells

associated with the lipophilic red dye. As shown in Figure 18, percent effector cells engaged in phagocytosis was on the order of 65% to 75%, depending on whether monocytes or neutrophils were tested. Increasing the bispecific antibody concentration above 1.0 μg/ml did not enhance the percent of effector cells engaged in phagocytosis.

Monocytes differentiated into macrophages (MDM) are known to mediate phagocytosis of tumor cells (Ely, P.*et al.* 1996, *Blood.* 87:3813-3821). To determine if A77X520C9 BsAb could induce phagocytosis by MDM, these effector cells were incubated with dye-labeled SKBR-3 cells in the presence of varying concentrations of BsAb. The level of phagocytosis was determined by 2-color flow cytometric analysis, in which one fluorescent label is denoted FL1 and the other is denoted FL2. Figure 19 shows that the MDM (panel 1, $FL1^+$, $FL2^-$) and SKBR-3 cells (panel 2, $FL1^-$, $FL2^+$) are distinguished from each other in a mixture of these two cell types by their unique fluorescence patterns (panel 3). When the A77X520C9 BsAb was added to the mixture of these target and effector cells, the BsAb mediated nearly a complete loss of tumor cells (panel 5, lower right quadrant). This was confirmed by an almost total lack of tumor cells that could be recovered from the BsAb containing wells as determined by tumor specific ELISA (data not shown). As expected, MDM alone mediated some phagocytosis (~45%) of SKBR-3 cells without the BsAb (panel 3). However, addition of 0.1 μg/ml A77X520C9 was sufficient to enhance the phagocytosis to >95% (panel 5). This BsAb-mediated phagocytic activity was almost completely inhibited by A77 $F(ab')_2$ (panel 6). Furthermore, a mixture of uncoupled A77 $F(ab')_2$ and 520C9 $F(ab')_2$ could not enhance phagocytosis (panel 4), indicating the need for a conjugated A77 F(ab')X 520C9 F(ab') BsAb to target tumor cells to effector cells leading to activation of the effector cells (MDM).

Figure 20 shows that the BsAb-mediated phagocytic activity was dose dependent and saturated (nearly complete loss of tumor cells) at 0.1 μg/ml of the BsAb. A77 $F(ab')_2$ almost completely blocked this BsAb-mediated phagocytosis. Again, a mixture of uncoupled A77 $F(ab')_2$ and 520C9 $F(ab')_2$ had no activity to mediate phagocytosis.

The above-described studies show that, in addition to promoting extracellular lysis, the FcαRI-directed BsAb mediated potent phagocytic activity. In particular, when MDM were used as effector cells, nearly 100% of tumor cells were phagocytosed. It is likely that BsAb-mediated both ADCC and phagocytic activities simultaneously, and entire cells or fragments of lysed cells were phagocytosed by the MDM via FcαRI. These two mechanisms have been shown to occur in concert for Fcγ RI-mediated phagocytosis (Ely, P.*et al.* 1996, *Blood.* 87:3813-3821). It has been shown

that antigen presenting phagocytic cells (such as MDM) can present antigens via both class I and class II pathways after phagocytosis of antigen bearing particles (Falo, L.D. *et al.* 1995. *Nature Medicine.* 1,7:649-656). Thus the potent phagocytosis of tumor cells via FcαRI can lead to activation of both humoral and cellular immune functions specifically directed to tumor associated antigens.

These cytotoxic activities of FcαRI-directed BsAb are of therapeutic value because FcαRI expression is limited primarily to immune effector cells that are herein demonstrated to mediate BsAb-dependent cytotoxic activities, *viz.*, PMN, monocytes and macrophages (Morton, H.C.*et al.* 1996.*Critical Reviews in Immunology.* 16:423). Because distribution of FcαRI is limited primarily to cytotoxic effector cells and because of its potent triggering activity, BsAb comprising a binding determinant to FcαRI can be of general use for cell-mediated immunotherapies. Furthermore, the approach described herein can be employed to prepare FcαRI-directed BsAb utilizing the existing high affinity tumor-specific IgG mAbs, that is, the binding determinant components of the anti-FcαRI BsAb can be IgG molecules, since they are converted into $F(ab')_2$ fragments prior to chemical coupling. Further, for construction of recombinant BsAb, the Fc portion of the IgG can be removed by appropriate restriction digestion and further exonuclease digestion as appropriate. The ability to use binding determinants from the Fab portion of IgG molecules will obviate the need to generate new IgA class tumor specific mAbs toexploit the cytotoxic potential of FcαRI.

Example 10: Phagocytosis of the fungal pathogen *Candida albicans*

Bispecific and multispecific antibody compositions of the invention can be used to direct FcαRI-bearing leukocytes against antigens from microbial pathogens, for example, against bacteria, viruses, protozoan and metazoan parasites, and fungi. By way of example, the following studies demonstrate that BsAb mediates phagocytosis of *Candida albicans*, a pathogenic yeast which causes deleterious infections in T-cell deficient immunocompromised patients.

A bispecific antibody directed against this pathogen was produced from rabbit polyclonal-anti *Candida* IgG (Biodesign, Kennebunk, ME). $F(ab')_2$ fragments of this polyclonal IgG were treated with sulfo-SMCC to add maleimide to free amino groups. This conjugate was reacted with equimolar A77 F(ab'), yielding the A77Xanti-*Candida* BsAb, which was purified from the uncoupled fragments by chromatography on Superdex 200 (Pharmacia, Piscataway, NJ). The A77Xanti-*Candida* bound effector and target cells consistent with the specificity of the component antibodies.

Candida albicans cells cultured overnight at 37°C were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS), and fluorescently labeled by incubation with FITC (Sigma, St. Louis, MO) at a concentration of 0.1 mg/ml in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 9.6) for 30 minutes at 4°C. Fluorescent fungal cells washed three times with PBS (5×10^5) were incubated for 30 minutes at 37°C with 2×10^5 isolated neutrophils in the presence of 10 μg/ml A77Xanti-*Candida* (A77 X α*Candida*) BsAb; controls were incubated without BsAb. Phagocytosis was quantitated by measuring FITC-fluorescence intensities of cells on a flow cytometer using PE-conjugated CD11b MoAb (Becton Dickinson, San Jose, CA) as a gate marker for neutrophils, and phagocytosis was determined also in cytospin preparations and evaluated by microscopy. Each experiment was repeated three times.

Phagocytosis of *Candida* by neutrophils, the most important effector cell population against pathogenic fungi such as *Candida* and *Aspergillus* species (Pizzo, P., 1984, Cancer <u>54</u>:2649; Schaffner, A. *et al.*, 1986, J. Clin. Invest. <u>78</u>:511), and the stimulation of phagocytosis by the bispecific antibody of the invention, are shown in Fig. 21. In the presence of A77 X anti-*Candida*, 37.3% ± 7.5 % of neutrophils were found to have phagocytosed fungal particles; in the absence of this BsAb 4.2 ± 1.5% of neutrophils had ingested fungal particles (average of three experiments). Thus the FcαRI BsAb caused 8.9-fold stimulation of neutrophil phagocytosis of the fungal pathogen as demonstrated photographically in panels A and B of Fig. 21, and by the shift in fluorescence intensity of the FITC-labeled fungal cells to that of PE-labeled neutrophils, in panels C and D.

FcαRI-directed BsAb can be used to combat a number of infectious diseases, since most infectious agents (bacteria, viruses, fungi etc.) express unique antigens on their surface and several pathogen-specific antibodies have been described. This technology can be applied to combat antibiotic resistant pathogens such as methicillin resistant *Staphylococcus aureus*, or other pathogenic bacterial species, and to pathogenic fungal species in addition to *Candida*.

Example 11: BsAb-Mediated Cytolysis With Effector Cells Treated With Cytokines and Growth Factors

Figure 22 shows percent cytotoxicity of SKBR-3 breast tumor target cells, as a function of concentration of A77X520C9 BsAb, by neutrophil effector cells pre-incubated with G-CSF (Panel A) or both G-CSF and IFN-γ (Panel B). Neutrophils were pre-incubated with these factors overnight at 37° prior to the cytotoxicity assay. In comparison with the data described in Example 4, in which neutrophils were not pre-incubated with cytokines, percent cytotoxicity was here found to be enhanced by pre-

incubation of effector cells with both G-CSF and IFN-γ, such that at 0.1 μg/ml BsAb, greater than 10% of tumor cells were specifically lysed compared to less than 10% seen in Example 1, and at 1.0 μg/ml BsAb, greater than 40% cytotoxicity was observed compared to approximately 24% to 32% in Figure 7 in the absence of these factors.

Figure 23 shows the results of pre-treating effector monocytes with IFN-γ (Panel B) or TNF (Panel C), using BsAb A77XH425, which combines the binding determinant for FcαR with the binding determinant for EGF-R. As described above, EGF receptors are known to be overexpressed on carcinomas of the breast, skin, head and neck and other tumor cells, so that BsAb A77XH425 comprises another embodiment of a multispecific composition for treatment of breast and other tumors. As shown in Figure 23, untreated monocytes caused substantial breast cancer cell cytotoxicity mediated by A77XH425 BsAb, at BsAb concentrations of 0.1 μg/ml and 1.0 μg/ml. Specifically, BsAb A77XH425 caused greater than 60% cytolysis at 1.0 μg/ml with untreated monocytes and with TNF treated monocytes, and greater than 40% at 1.0 μg/ml with IFN-γ-treated monocytes.

Overall, these results demonstrate that each of the two BsAb preparations, A77X520C9 and A77XH425, bind to breast tumor cells or skin carcinoma cells, respectively, and mediate cytolysis of the cancer cells by neutrophils or monocytes without additional treatment of these effector cells by exogenous cytokines.

Example 12: The A77 FcαR Binding Site is Different From That of FcαR for its Natural Ligand

In design of a therapeutic BsAb directed to FcαRI, optimal functionality as a therapeutic agent in subjects would be achieved if binding of this entity were independent of competition with endogenous molecular species, such as the natural ligand IgA. Thus a therapeutic BsAb or multispecific binding composition could be administered, and not be blocked or inhibited, or not be substantially blocked or inhibited, by endogenous IgA.

Figure 24 shows that A77 antibody bound to the full extent to effector cells in the presence of IgA at 200 μg/ml, compared to control binding in the absence of IgA. Mean fluorescence intensity was unaffected by the presence of IgA. These results demonstrate that A77 mAb specifically binds an epitope on FcαRI that is different from the site for binding of IgA (Monteiro *et al.* 1992, *J. Immunol. 148*: 1764-1770).

Example 13: FcαRI Modulation by A77, and Absence of Modulation by A77X520C9 and A77 F(ab')$_2$

Characterization of the effect of addition of A77 mAb to cells on FcαRI regulation was achieved by studies in which modulation (decrease in receptor number) of FcαRI from the cell surface was examined as a function of concentration of A77 mAb.

As shown in Figure 25, incubation of monocytes with various concentrations of A77 for 18h at 37°C caused modulation at 10 nanograms /ml, which reached a plateau at 55% to 60% of control (number of receptors in the absence of A77) at 1.0 to 10 μg/ml. In contrast, incubation of monocytes with antibody 520C9, which has the same isotype as A77 and which specifically binds the HER2/*neu* receptor that is not expressed on monocytes, had no effect on monocyte modulation of FcαR. Thus the A77 mAb functional determinant is capable of causing internalization and modulation of FcαR from the surface. Further, ability of the BsAb to bind HER2/*neu* by virtue of a binding determinant derived from 520C9 is independent of the FcαRI binding determinant. This result shows that down modulation of FcαR is achieved by incubation of cells bearing this receptor with antibody A77. Such modulation can be used for regulation of autoimmune disorders.

These findings are confirmed and contrasted with data for the BsAb A77X520C9, as well as A77 F(ab')$_2$, which show no down modulation of FcαRI in monocytes, and little in PMN compared to that of A77. The modulation of FcαRI expression upon BsAb or A77 mAb binding to monocytes or PMN was examined by flow cytometry. Figure 26 shows that 1 and 10 μg/ml whole A77 mAb induced about 40%-50% reduction of FcαRI on PMN and monocytes after overnight incubation at 37^0 C. This modulation activity did not require cross-linking of the bound A77 by an anti-murine antibody. However, 1 and 10 μg/ml of A77X520C9 BsAb or A77 F(ab')$_2$ induced minimum or no modulation of FcαRI under the similar conditions, indicating that the Fc region of the A77 mAb may be required to down modulate FcαRI expression.

From the data of Figure 26 showing modulation by A77 mAb but not by BsAb or A77 F(ab')$_2$, it can be concluded that BsAb binding to monocytes and PMN does not lead to cross-linking and subsequent down modulation of FcaRI in the absence of target antigen or cells. Therefore, the FcαRI-directed BsAb can be used to "arm" the effector cells in a subject or a patient, without activation by receptor cross-linking, thus avoiding undesired systemic side effects. Such effector cells armed with multispecific antibodies of the invention can be activated locally only upon cross-linking of FcαRI by binding a target antigen, for example, an antigen on a tumor cell or on a pathogen. Similar arming of monocytes by FcγRI-directed BsAb has been shown (Valone, F.H.*et*

al. 1995. *J. Clin. Oncol.* 13:2281-2292), however, for FcγRI-directed BsAb arming to be effective, pretreatment of the subject with G-GSF or IFN-γ is required to engage the PMN effector population *in vivo* (Repp, R.*et al.* 1991. *Blood.* 78:995; Weber, J.S.*et al.* 1996. *Proc. of ASCO.* 15:354. (Abstr.) FcαRI-directed BsAb described here could engage effector cells such as monocytes, PMN and macrophages without cytokine pretreatment, and without down-modulation of the cognate receptor on the leukocytes.

Example 14: Cloning and Sequencing A77 Variable Region Genes

A77 RNA was prepared from A77 FcαR specific antibody producing hybridoma cells, and 33 μg of total RNA was obtained from approximately 4 x 10^7 A77 cells using the RNAeasy Total RNA kit (Qiagen). RT-PCR was then done on 200 ng of the total RNA preparation using the GeneAmp® Thermostable r*Tth* Reverse Transcriptase RNA PCR kit (Perkin Elmer). Ig V region cDNAs were made using primers CG1FOR, 5'-GG*AAGCTT*AGACAGATGGGGGTGTCGTTTTG, SEQ ID NO: 1 (encoding amino acids 115-122 of the murine IgG1 heavy chain CH1 domain and a *Hin*dIII site) and CK2FOR,5'-GG*AAGCTT*GAAGATGGATACAGTTGGTGCAGC, SEQ ID NO: 2 (encoding amino acids 111-118 of the murine κ light chain constant domain and a *Hin*dIII site).

The V_H and V_κ cDNAs were amplified by PCR using the cDNA primers along with SH1BACKBAM, 5'-GACT*GGATCC*ATGGRATGGAGCTGGRTCWTBHTCTT, SEQ ID NO: 3 (encoding a consensus sequence of amino acids -20 to -12 of some V_H signal peptides and a *Bam*HI site) and VK1BACKBAM, 5'-GACT*GGATCC*GACATTCAGCTGACCCAGTCTCCA, SEQ ID NO: 4 (encoding amino acids -4 to -1 of the signal peptide and residues 1 to 4 of some murine V_κ domains and a *Bam*HI site). The single-letter code for combinations of nucleotides, known to those of skill in the art, is given on p.174 of the 1996-1997 New England Biolabs catalog (32 Tozer Rd., Beverly, MA).

Amplified V_H and V_κ DNA were purified using Wizard PCR Prep kit (Promega), cloned into pUC19, and sequenced by the dideoxy method. DNA sequencing was carried out by National Biosciences, Inc. At least 5 pUC19 clones of each V_κ and V_H were sequenced to obtain consensus sequences of these genes, which are shown in Figs. 27 (SEQ ID NO: 5) and 28 (SEQ ID NO: 7). The predicted amino acid sequences are shown in Figs. 27 (SEQ ID NO: 6) and 28 (SEQ ID NO: 8) for V_κ and V_H respectively.

These sequences are used to obtain recombinant humanized A77 FcαR-binding determinants, to produce single-chain antibodies and single-chain BsAbs, for engineering determinants with greater affinity using recombinant methods, for modeling studies to develop mimetic drugs using rational drug design, and for additional applications described in the instant invention.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

The contents of all patents and publications referred to herein are hereby incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MEDAREX, INC.
(B) STREET: 1545 ROUTE 22 EAST, P.O. BOX 992
(C) CITY: ANNANDALE
(D) STATE: NEW JERSEY
(E) COUNTRY: US
(F) POSTAL CODE: 08801-0992
(G) TELEPHONE:
(H) TELEFAX:

(ii) TITLE OF INVENTION: THERAPEUTIC MULTISPECIFIC COMPOUNDS COMPRISED OF ANTI-FCα RECEPTOR ANTIBODIES

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LAHIVE & COCKFIELD, LLP
(B) STREET: 60 State Street, Suite 510
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: US
(F) ZIP: 02109-1875

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII TEXT

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US97/
(B) FILING DATE: 10 JULY 1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/678,194
(B) FILING DATE: 11 JULY 1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: REMILLARD, JANE E.
(B) REGISTRATION NUMBER: 38,872
(C) REFERENCE/DOCKET NUMBER: MXI-064CPPC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617)227-7400
(B) TELEFAX: (617)227-5941

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..31
(D) OTHER INFORMATION: /note= "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGAAGCTTAG ACAGATGGGG GTGTCGTTTT G 31

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..32
(D) OTHER INFORMATION: /note= "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAAGCTTGA AGATGGATAC AGTTGGTGCA GC 32

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..36
(D) OTHER INFORMATION: /note= "PCR primer"

(vii) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 15,26
(D) OTHER INFORMATION: /note= "R is A or G"

(viii) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 29
(D) OTHER INFORMATION: /note= "W is A or T"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 31
(D) OTHER INFORMATION: /note= "B is C or G or T"

(x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 32
(D) OTHER INFORMATION: /note= "H is A or C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACTGGATCC ATGGRATGGA GCTGGRTCWT BHTCTT 36

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..34
(D) OTHER INFORMATION: /note= "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACTGGATCC GACATTCAGC TGACCCAGTC TCCA 34

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..336

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GAC ATT CAG CTG ACC CAG TCT CCA CTC ACT TTG TCG ATT ACC ATT GGA      48
Asp Ile Gln Leu Thr Gln Ser Pro Leu Thr Leu Ser Ile Thr Ile Gly
  1               5                  10                  15

CAA CCA GCC TCC ATC TCT TGC AAG TCA AGT CAG AGC CTC TTA GAT AGT      96
Gln Pro Ala Ser Ile Ser Cys Lys Ser Ser Gln Ser Leu Leu Asp Ser
             20                  25                  30

GAT GGA AAG ACA TAT TTG AAT TGG TTG TTA CAG AGG CCA GGC CAG TCT     144
Asp Gly Lys Thr Tyr Leu Asn Trp Leu Leu Gln Arg Pro Gly Gln Ser
         35                  40                  45

CCA ACG CGC CTA ATC TAT CTG GTG TCT AAA CTG GAC TCT GGA GTC CCT     192
Pro Thr Arg Leu Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro
     50                  55                  60

GAC AGG TTC ACT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTG AAA ATC     240
Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65                  70                  75                  80

AGC AGA GTG GAG GCT GAG GAT TTG GGA ATT TAT TAT TGC TGG CAA GGT     288
Ser Arg Val Glu Ala Glu Asp Leu Gly Ile Tyr Tyr Cys Trp Gln Gly
                 85                  90                  95

GCA CAT TTT CCT CAG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA     336
Ala His Phe Pro Gln Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Asp Ile Gln Leu Thr Gln Ser Pro Leu Thr Leu Ser Ile Thr Ile Gly
  1               5                  10                  15

Gln Pro Ala Ser Ile Ser Cys Lys Ser Ser Gln Ser Leu Leu Asp Ser
             20                  25                  30

Asp Gly Lys Thr Tyr Leu Asn Trp Leu Leu Gln Arg Pro Gly Gln Ser
         35                  40                  45

Pro Thr Arg Leu Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro
     50                  55                  60

Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Leu Gly Ile Tyr Tyr Cys Trp Gln Gly
                 85                  90                  95

Ala His Phe Pro Gln Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 426 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..426

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG GGA TGG AGC TGG GTC ATT ATC TTC CTC CTG TCA GGA ACT GCA GGA      48
Met Gly Trp Ser Trp Val Ile Ile Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

GCC CAC TCT GAG ATC CAG CTG CAG CAG ACT GGA CCT GAG CTG GTG AAG      96
Ala His Ser Glu Ile Gln Leu Gln Gln Thr Gly Pro Glu Leu Val Lys
            20                  25                  30

CCT GGG GCT TCA GTG AAG ATA TCC TGC AAG GCT TCT GGT TAT TCA TTC     144
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe
        35                  40                  45

ACT GAC TAC ATC ATA TTT TGG GTG AAG CAG AGC CAT GGA AAG AGC CTT     192
Thr Asp Tyr Ile Ile Phe Trp Val Lys Gln Ser His Gly Lys Ser Leu
    50                  55                  60

GAG TGG ACT GGA AAT ATT AAT CCT TAC TAT GGT AGT ACT AGC TAC AAT     240
Glu Trp Thr Gly Asn Ile Asn Pro Tyr Tyr Gly Ser Thr Ser Tyr Asn
65                  70                  75                  80

CTG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA GAC AAA TCT TCC AGC     288
Leu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
                85                  90                  95

ACA GCC TAC ATG CAG CTC AAC AGT CTG ACA TCT GAG GAC TCT GCA GTC     336
Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

TAT TAC TGT GTA AGA GGA GTT TAT TAC TAC GGT AGT AGC TAC GAG GCG     384
Tyr Tyr Cys Val Arg Gly Val Tyr Tyr Tyr Gly Ser Ser Tyr Glu Ala
        115                 120                 125

TTT CCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA             426
Phe Pro Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 142 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Gly Trp Ser Trp Val Ile Ile Phe Leu Leu Ser Gly Thr Ala Gly
 1               5                  10                  15

Ala His Ser Glu Ile Gln Leu Gln Gln Thr Gly Pro Glu Leu Val Lys
            20                  25                  30
```

```
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe
        35                  40                  45

Thr Asp Tyr Ile Ile Phe Trp Val Lys Gln Ser His Gly Lys Ser Leu
    50                  55                  60

Glu Trp Thr Gly Asn Ile Asn Pro Tyr Tyr Gly Ser Thr Ser Tyr Asn
65                  70                  75                  80

Leu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
                85                  90                  95

Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

Tyr Tyr Cys Val Arg Gly Val Tyr Tyr Tyr Gly Ser Ser Tyr Glu Ala
        115                 120                 125

Phe Pro Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
    130                 135                 140
```

What is claimed is:

1. A bispecific binding molecule, comprising a first binding determinant which binds to an Fcα receptor without being blocked by immunoglobulin A, and a second binding determinant which binds to a target antigen.

2. The bispecific binding molecule of claim 1, wherein the binding of the first binding determinant to Fcα receptor is not blocked by human immunoglobin A.

3. The bispecific binding molecule of claim 1, wherein the target antigen is a cancer cell antigen.

4. The bispecific binding molecule of claim 3, wherein the cancer cell antigen is selected from the group consisting of cancers of the breast, ovary, testis, lung, colon, rectum, pancreas, liver, central nervous system, head and neck, kidney, bone, blood and lymphatic system.

5. The bispecific binding molecule of claim 1, wherein the target antigen is an infectious disease antigen from a pathogen or pathogen-infected cell.

6. The bispecific binding molecule of claim 3, wherein the cancer cell antigen is a member of the human EGF-like receptor family.

7. The bispecific binding molecule of claim 6, wherein the cancer cell antigen is an EGF receptor.

8. The bispecific binding molecule of claim 6, wherein the cancer cell antigen is HER-2/*neu*.

9. The bispecific binding molecule of claim 6, wherein the cancer cell antigen is selected from the group consisting of HER-3 and HER-4.

10. The bispecific binding molecule of claim 6, wherein the cancer cell antigen is a heterodimeric receptor comprised of at least one HER subunit.

11. The bispecific binding molecule of claim 3, wherein the cancer cell antigen is selected from the group consisting of carcinoembryonic antigen, gastrin releasing peptide receptor antigen, and mucine tumor antigen TAG 72.

12. The bispecific molecule of claim 1, wherein at least one of said binding determinants is an antibody or an antibody fragment.

13. The bispecific binding molecule of claim 12, wherein the antibody or antibody fragment is an IgG or IgG fragment.

14. The bispecific binding molecule of claim 12, wherein the antibody fragment is selected from the group consisting of an Fab, Fab', $F(ab')_2$, Fv, and single chain Fv.

15. A bispecific binding molecule comprising a binding determinant which binds to an Fcα receptor without being blocked by immunoglobulin A and a binding determinant for a target selected from the group consisting of a cancer cell antigen and an antigen from a pathogen.

16. The bispecific binding molecule of claim 15, wherein the determinant which binds an Fcα receptor is humanized.

17. The bispecific binding molecule of claim 16, wherein the humanized determinant is derived from all or a portion of the nucleotide sequences encoding the Fv binding determinants of antibody A77 V_K and V_H regions shown in Figure 14 (SEQ ID NO:5), and Figure 15 (SEQ ID NO:7), respectively.

18. The bispecific binding molecule of claim 16, wherein the humanized determinant is derived from all or a portion of the amino acid residue sequences of the Fv binding determinants of antibody A77 V_K and V_H regions shown in Figure 14 (SEQ ID NO:6), and Figure 15 (SEQ ID NO:8), respectively.

19. The bispecific binding molecule of claim 18, wherein the humanized determinant is greater than 50% homologous to that of antibody A77 V_K or V_H regions shown in Figure 14 (SEQ ID NO:6), and Figure 15 (SEQ ID NO:8), respectively.

20. The bispecific binding molecule of claim 15, wherein the second binding determinant is selected from the group consisting of ATCC HB 8696 (antibody 520C9), ATCC HB 9459 (antibody CC49) and functional fragments thereof.

21. The bispecific binding molecule of claim 20 produced by chemical linkage of the antibody A77 fragment and the second binding determinant.

22. The bispecific binding molecule of claim 20 which is produced recombinantly in a host cell.

23. A multispecific binding molecule, comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds one or more target antigens.

24. The multispecific binding molecule of claim 23, wherein the binding of the first binding determinant to Fcα receptor is not blocked by human immunoglobin A.

25. The multispecific binding molecule of claim 23 selected from the group consisting of a bispecific binding molecule and a trispecific binding molecule.

26. The multispecific binding molecule of claim 25, comprising a third binding determinant specific for an Fc receptor that is not an Fcα receptor.

27. The multispecific binding molecule of claim 26, wherein the third binding determinant binds an Fc receptor selected from the group consisting of Fcγ receptor, Fcε receptor, Fcδ receptor and Fcμ receptor.

28. The multispecific binding molecule of claim 23, wherein binding of the Fcγ receptor is not inhibited by human IgG.

29. The multispecific binding molecule of claim 28, wherein the second binding determinant binds to a target antigen selected from the group consisting of a cancer cell antigen, a pathogen antigen, and an antigen on a pathogen-infected cell.

30. The multispecific binding molecule of claim 28 produced by chemical linkage of said binding determinants.

31. The multispecific binding molecule of claim 23 produced recombinantly in a host cell transfected with a nucleic acid encoding said binding determinants.

32. The multispecific molecule of claim 23, wherein at least one of said binding determinants is an antibody or an antibody fragment.

33. The multispecific molecule of claim 32, wherein at least one of said binding determinants is a humanized antibody or fragment thereof.

34. The multispecific binding molecule of claim 29, wherein the cancer cell antigen is from a cancer cell selected from the group consisting of cancer cells of the breast, ovary, testis, lung, colon, rectum, pancreas, liver, central nervous system, head and neck, kidney, bone, blood and lymphatic system.

35. The multispecific binding molecule of claim 29, wherein the pathogen antigen is selected from the group consisting of bacterial, fungal, protozoal, and viral antigens.

36. The multispecific binding molecule of claim 29, wherein the cancer cell antigen is a member of the human EGF-like receptor family.

37. The multispecific binding molecule of claim 36, wherein the cancer cell antigen is an EGF receptor.

38. The multispecific binding molecule of claim 36, wherein the cancer cell antigen is HER-2/*neu*.

39. The multispecific binding molecule of claim 36, wherein the cancer cell antigen is HER-3, HER-4, or a heteromultimeric receptor comprised of at least one HER subunit.

40. The multispecific binding molecule of claim 29, wherein the cancer cell antigen is selected from the group consisting of carcinoembryonic antigen, gastrin releasing peptide receptor antigen, and mucine antigen TAG 72.

41. A multispecific binding molecule comprising a functional fragment of antibody A77 and a second binding determinant which binds an antigen selected from the group consisting of an antigen on a cancer cell, an antigen on a pathogen and an antigen on a pathogen-infected cell.

42. The multispecific binding molecule of claim 41, wherein the second binding determinant for an antigen on a cancer cell is selected from the group consisting of ATCC HB 8696 (antibody 520C9), ATCC HB 9459 (antibody CC49), and functional fragments thereof.

43. The multispecific binding molecule of claim 41, wherein the A77 antibody fragment binds to an IgA receptor on a white blood cell.

44. The multispecific binding molecule of claim 43, wherein the white blood cell is selected from the group consisting of a macrophage, monocyte, neutrophil, basophil, eosinophil, and lymphocyte.

45. The multispecific binding molecule of claim 41, wherein the pathogen is selected from the group consisting of bacteria, viruses, fungi and protozoans.

46. The multispecific binding molecule of claim 32, wherein the antibody or antibody fragment is an IgG or IgG fragment.

47. The multispecific binding molecule of claim 32, wherein the antibody fragment is selected from the group consisting of an Fab, Fab', $F(ab')_2$, Fv, and single chain Fv.

48. The multispecific binding molecule of claim 23 comprising a third binding determinant which binds to an antigen of a target cell, wherein said second binding determinant binds to a different antigen on the same target cell.

49. The multispecific binding molecule of claim 23, comprising a third binding determinant which binds to an epitope of a target antigen, wherein said second binding determinant binds to a different epitope on the same target antigen.

50. The multispecific binding molecule of claim 45, wherein the pathogen is a fungus.

51. The multispecific binding molecule of claim 50, wherein the fungus is a species of the genus *Candida*.

52. The multispecific binding molecule of claim 51, wherein the species is *C. albicans*.

53. The multispecific molecule of claim 50, wherein the binding determinant to Fcα is not blocked by human immunoglobin A.

54. A method for eliminating or reducing an unwanted cell in a subject, comprising administering to the subject a therapeutically effective dose of a multispecific binding molecule, comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds an antigen on the unwanted cell, in a pharmaceutically acceptable carrier.

55. The method of claim 54, wherein at least one of said binding determinants is humanized.

56. The method of claim 55, further comprising administering to the subject at least one agent that enhances the number or activity of Fcα receptors on an Fcα receptor-bearing cell.

57. The method of claim 56, wherein the agent is a cytokine.

58. The method of claim 57, wherein the cytokine is selected from the group consisting of at least one of G-CSF, GM-CSF, IFN-γ, and TNF.

59. A method for treating a subject infected with a pathogen wherein the subject is administered a therapeutically effective dose of a multispecific binding molecule, said molecule comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds a target antigen of the pathogen or a pathogen-infected cell, in a pharmaceutically acceptable carrier.

60. A method for treating a subject to remove an unwanted cell in the subject, comprising

obtaining a sample of blood or blood cells from the subject;

contacting said blood or blood cells *ex vivo* with a therapeutically effective dose of a multispecific binding molecule, said binding molecule comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds one or more target antigens, in a pharmaceutically acceptable carrier; and

returning said treated blood or blood cells to the subject.

61. The method of claim 60, wherein at least one of said determinants is humanized.

62. The method of claim 61, wherein said blood cells are isolated and expanded in culture.

63. The method of claim 61 for treating a subject for the presence of an unwanted cell, wherein said blood cells are treated with at least one agent that enhances the number or activity of Fcα receptors.

64. A method for vaccinating a subject against a pathogen or against a cancer, wherein the subject is administered a multispecific binding molecule, said molecule comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds an antigen selected from the group consisting of an antigen on a cancer cell, an antigen on a pathogen and an antigen on a pathogen-infected cell, in a pharmaceutically acceptable carrier.

65. A method for arming effector cells of a subject against a pathogen or against a cancer, wherein the subject is administered a multispecific binding molecule, said molecule comprising a first binding determinant which binds an Fcα receptor and a second binding determinant which binds an antigen selected from the group consisting of an antigen on a cancer cell, an antigen on a pathogen and an antigen on a pathogen-infected cell, in a pharmaceutically acceptable carrier.

66. A method for modulating an Fcα receptor in a subject, comprising administering to the subject a composition comprising antibody A77 or a functional fragment of A77, in a pharmaceutically acceptable carrier.

67. A method for identifying for an agent which modulates Fcα receptors on the surface of cells, comprising

contacting a sample of cells carrying Fcα receptors with the agent;

determining Fcα receptor activity in the sample with the agent, in a control sample with an antibody that modulates Fcα receptors, and in a control sample with cells not contacted with said agent or with antibody; and

comparing Fcα receptor activities in the samples, such that a sample of cells contacted with said agent and having statistically significant less Fcα receptor activity than control cells not contacted with agent, or having statistically significantly as low Fcα receptor activity as cells in a sample with an antibody, identifies an agent which modulates Fcα receptors on the surface of cells.

68. A method for designing an agent which modulates Fcα receptors for treatment of autoimmune disease by obtaining a three dimensional model of the A77 anti-Fcα receptor binding site using the sequence determinants of A77 heavy and light chain variable regions, comprising

comparing the amino acid residues of the A77 variable region with that of heavy and light chain variable regions of antibodies of known three dimensional structure;

determining placement of non-homologous amino acid residues within the main peptide chain of the binding region of the V_H and V_K sites, such that the size, shape and charge of the A77 anti-Fcα receptor binding site is determined;

screening a library of molecules to obtain those of suitable size, shape and charge by computer modeling that are mimetics of the A77 binding site; and

screening such candidates of appropriate size, shape and charge for activity as potential modulators of Fcα receptors, such that an agent which modulates Fcα receptors is designed.

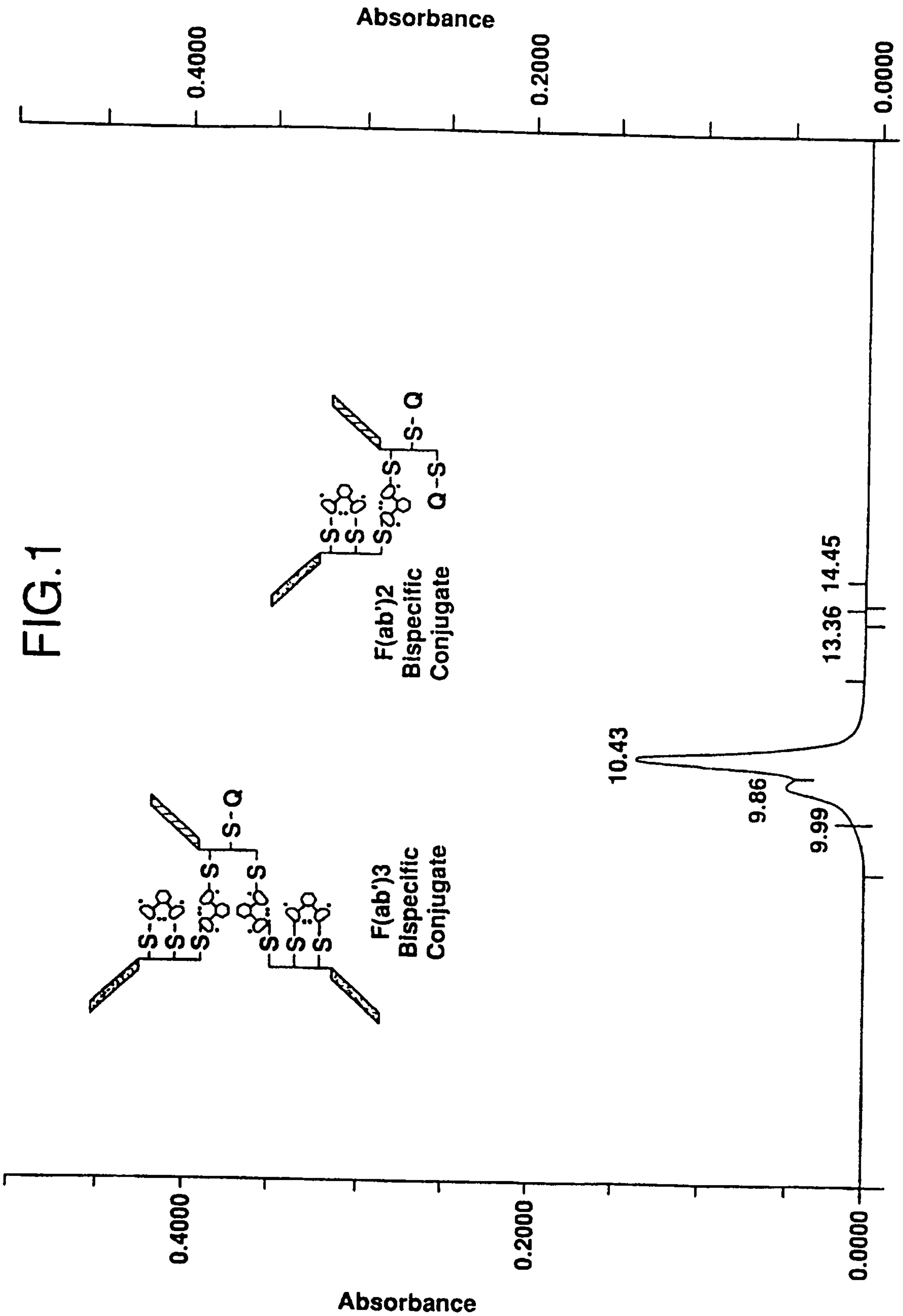

Absorbance
0.4000
0.2000
0.0000
10.43
9.86
9.99
13.36
14.45
F(ab')2
Bispecific
Conjugate
F(ab')3
Bispecific
Conjugate
S-Q
Q-S

FIG.1

FIG.2

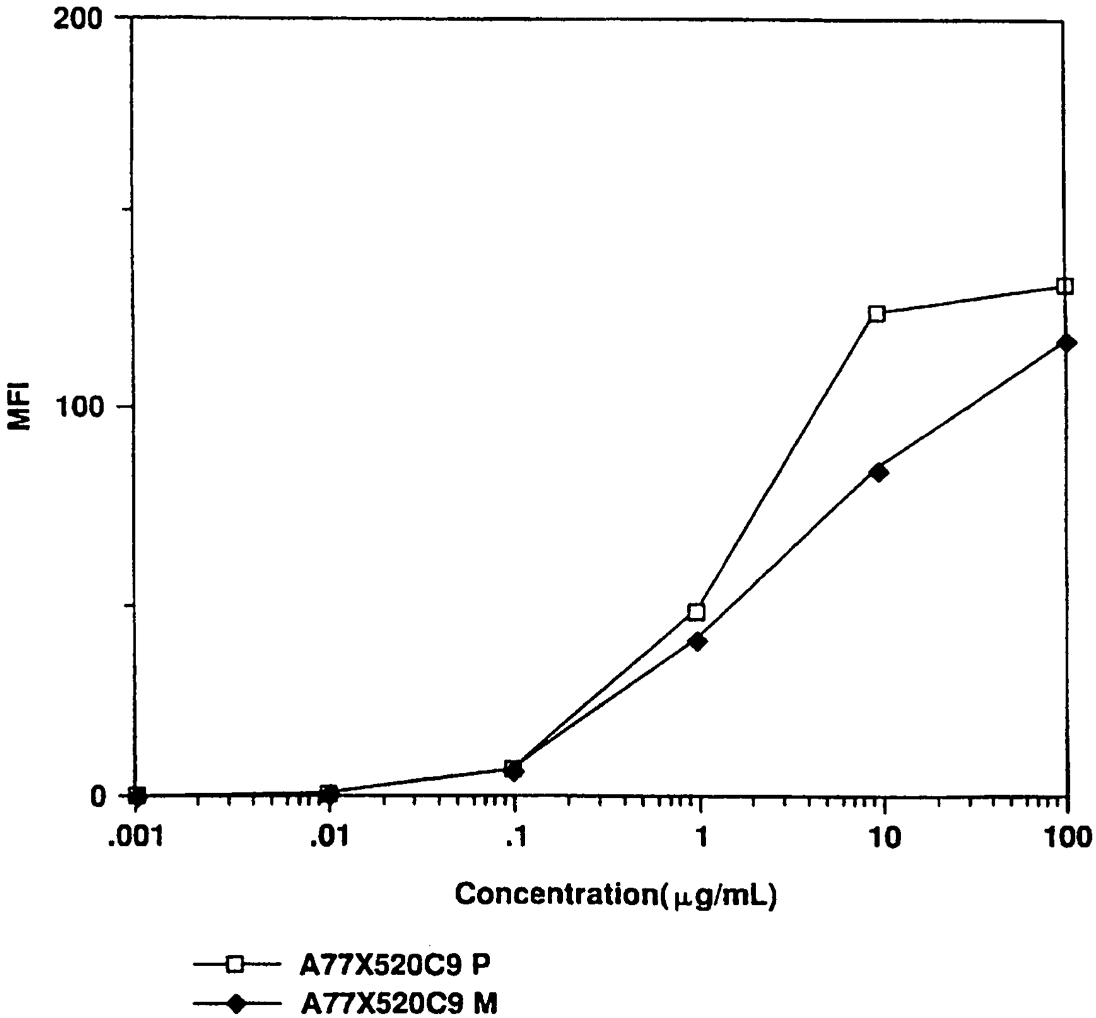

200
100
0
MFI
.001
.01
.1
1
10
100
Concentration(μg/mL)
A77X520C9 P
A77X520C9 M

FIG.3A

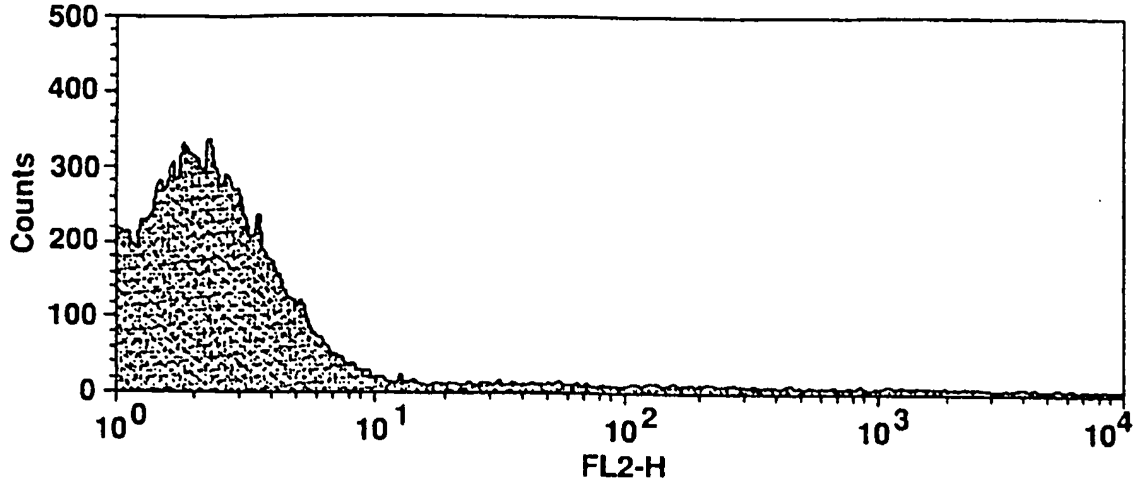


FIG.3B

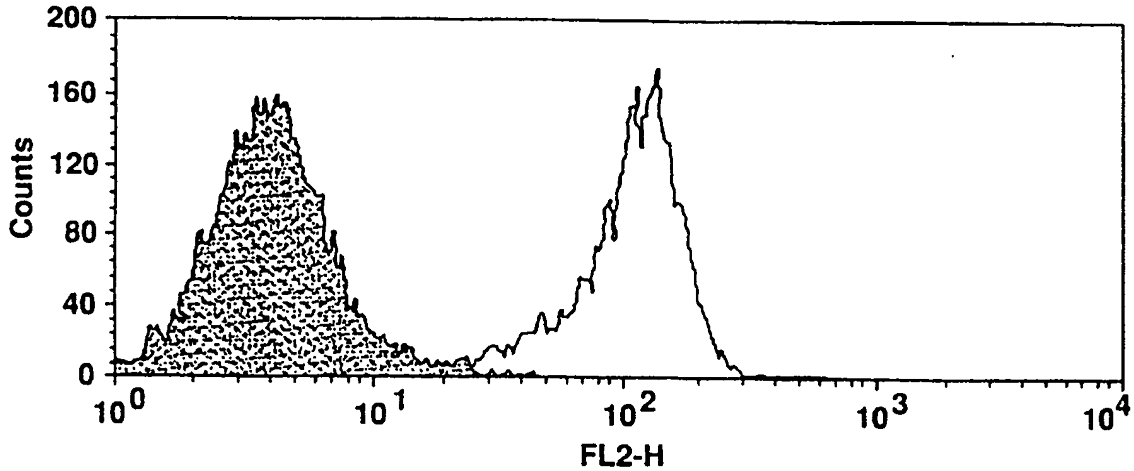


FIG.3C

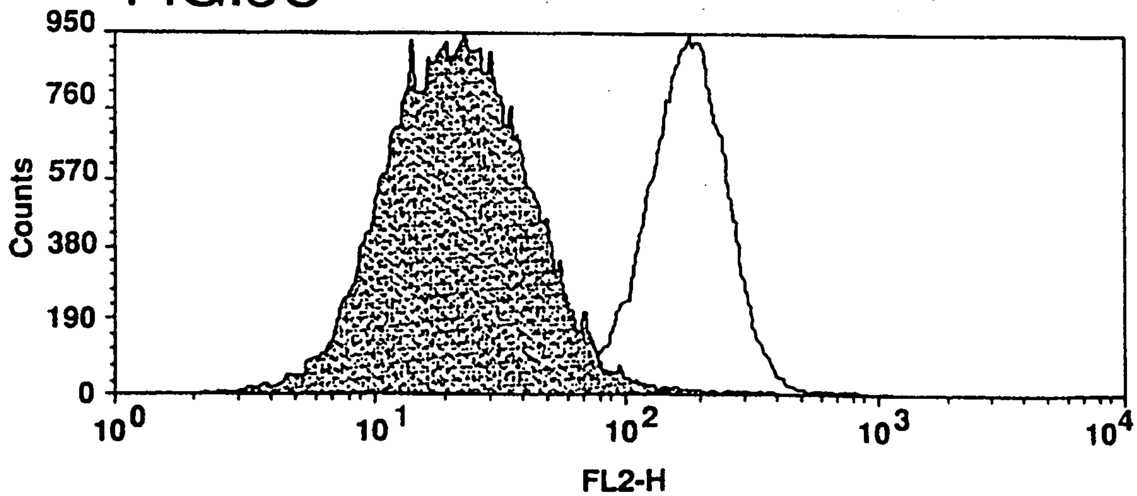


FIG.4

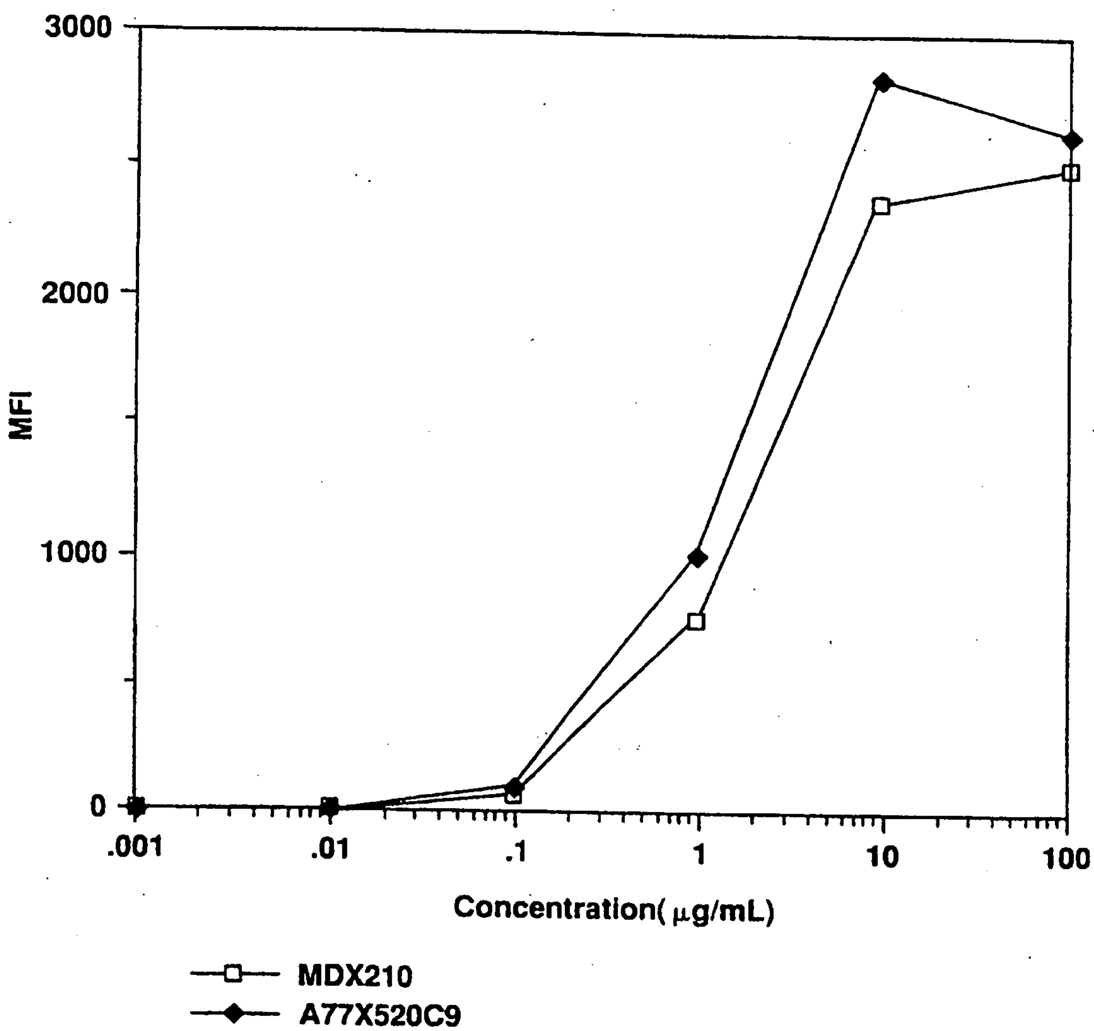

3000
2000
1000
0
MFI
.001
.01
.1
1
10
100
Concentration(μg/mL)
MDX210
A77X520C9

FIG.5

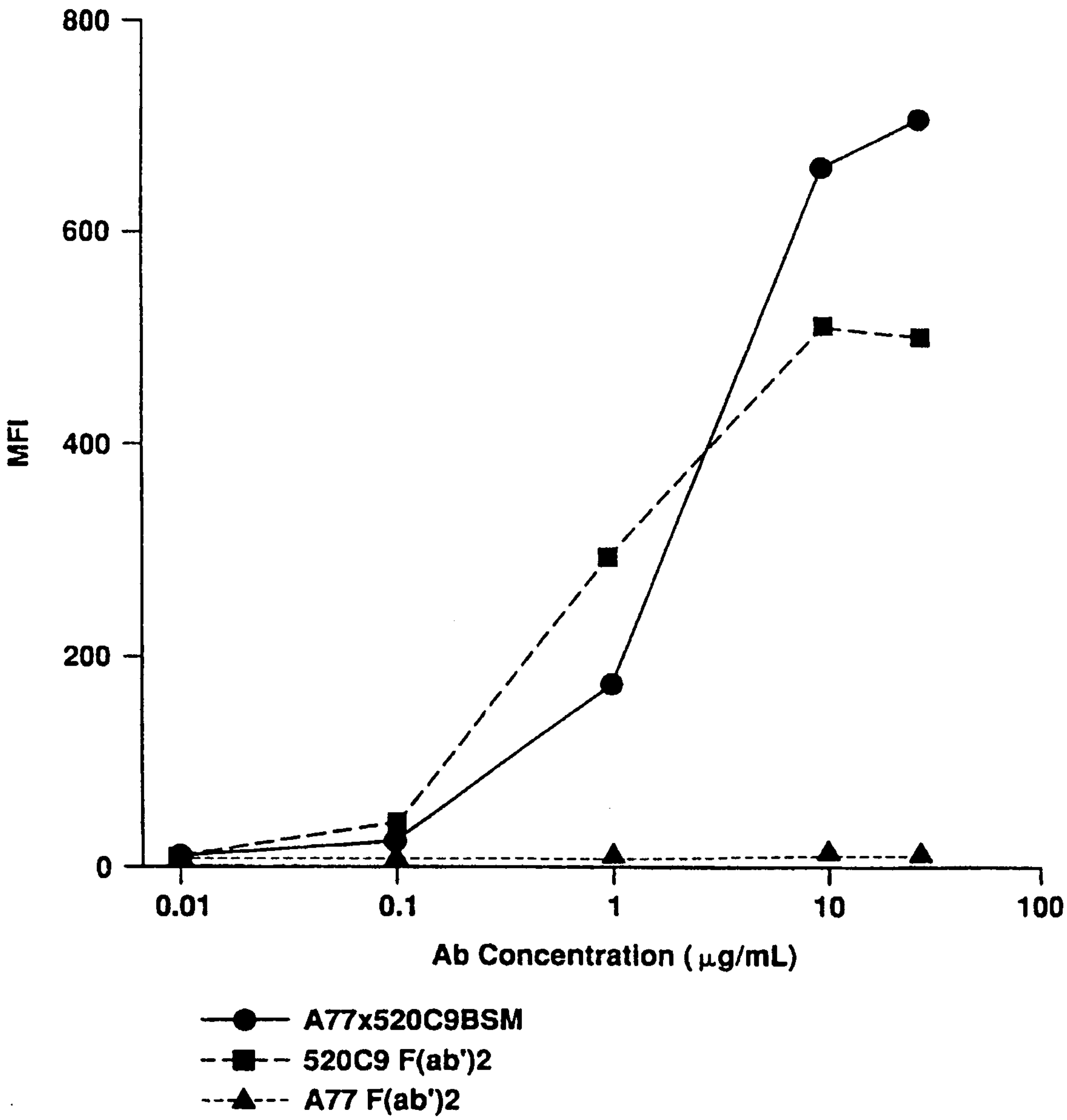

800
600
400
200
0
MFI
0.01
0.1
1
10
100
Ab Concentration (μg/mL)
A77x520C9BSM
520C9 F(ab')2
A77 F(ab')2

FIG.6

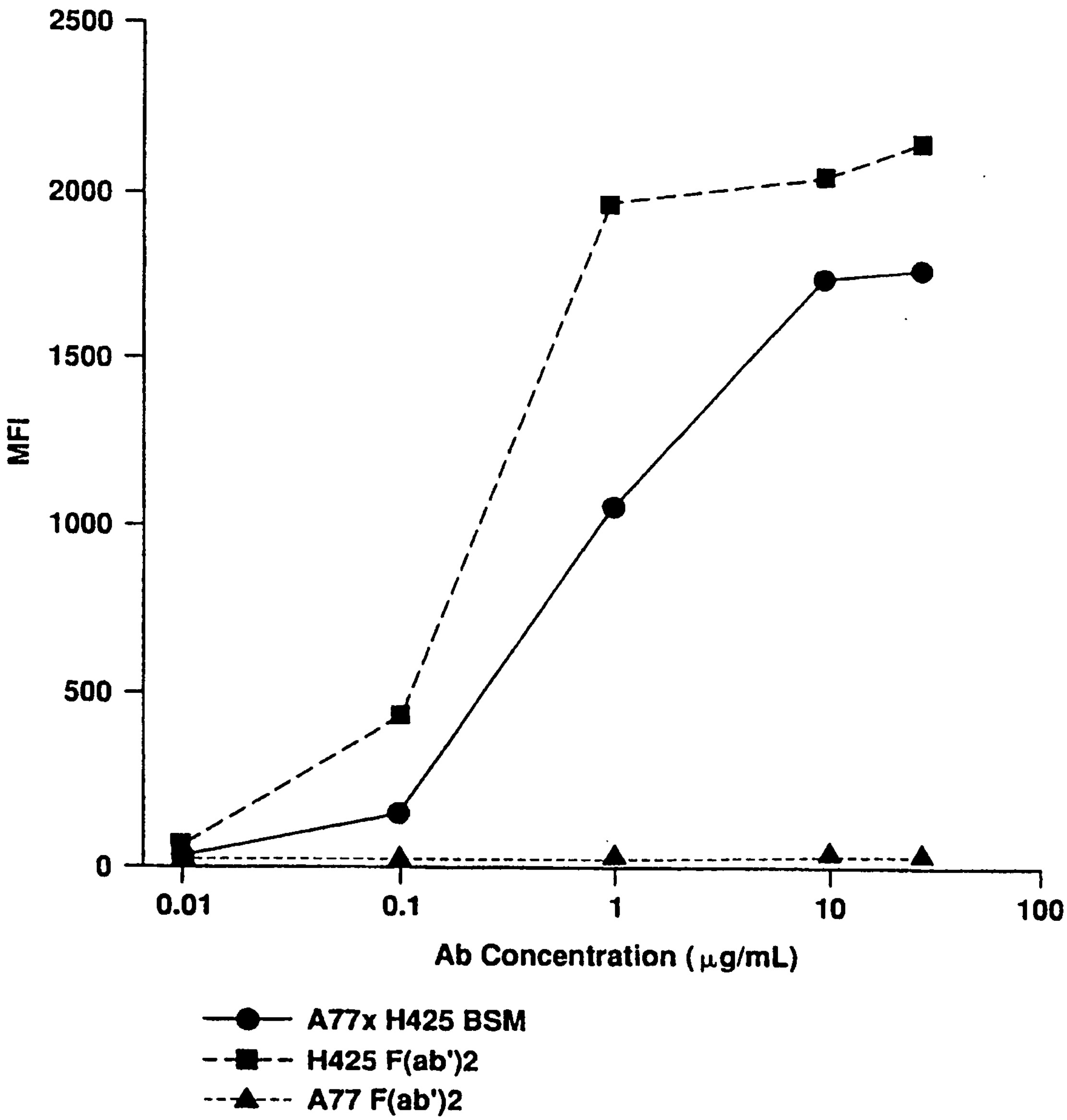

2500
2000
1500
1000
500
0
MFI
0.01
0.1
1
10
100
Ab Concentration (μg/mL)
A77x H425 BSM
H425 F(ab')2
A77 F(ab')2

FIG.7

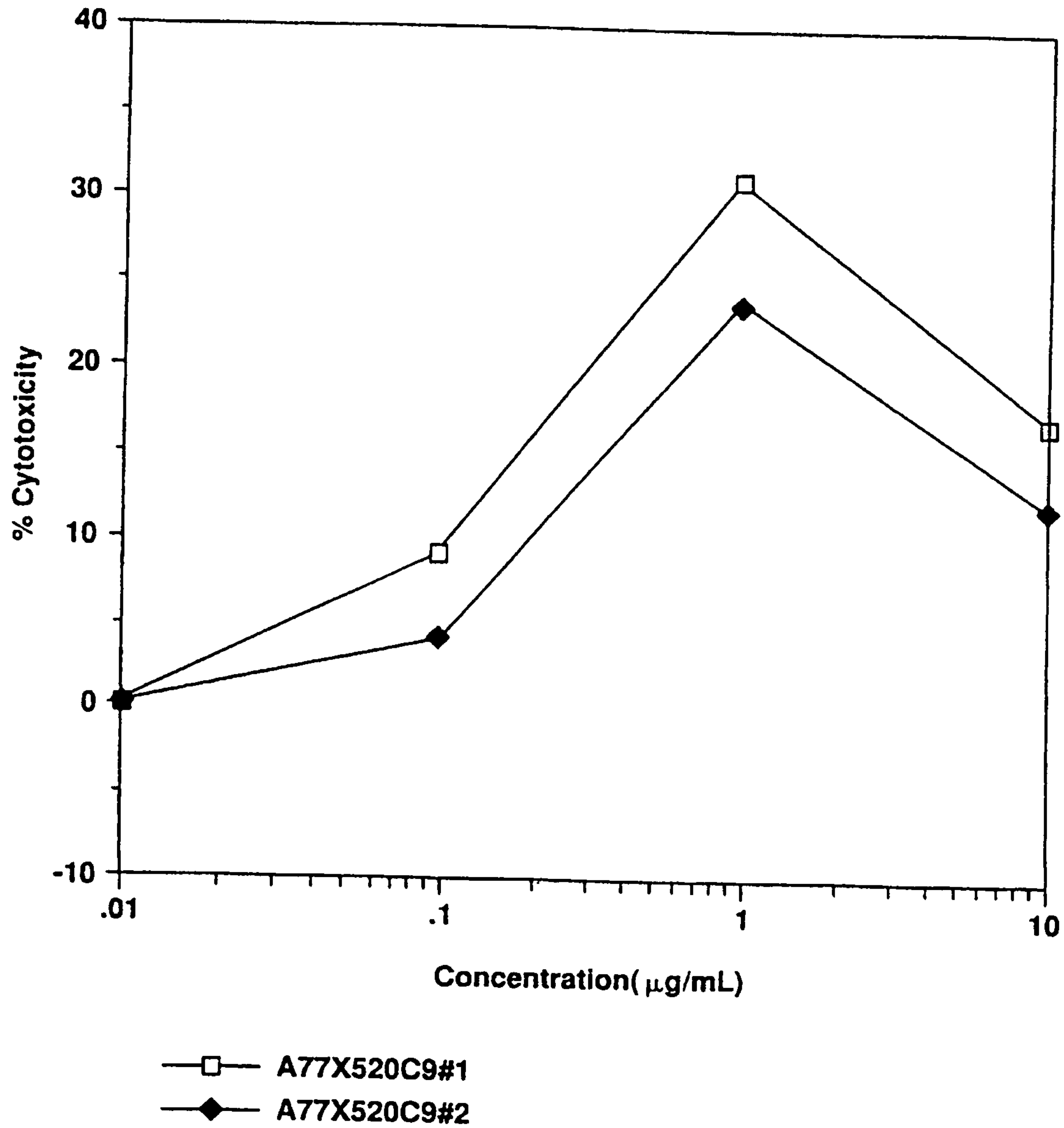

40
30
20
10
0
-10
% Cytotoxicity
.01
.1
1
10
Concentration(μg/mL)
A77X520C9#1
A77X520C9#2

FIG.8

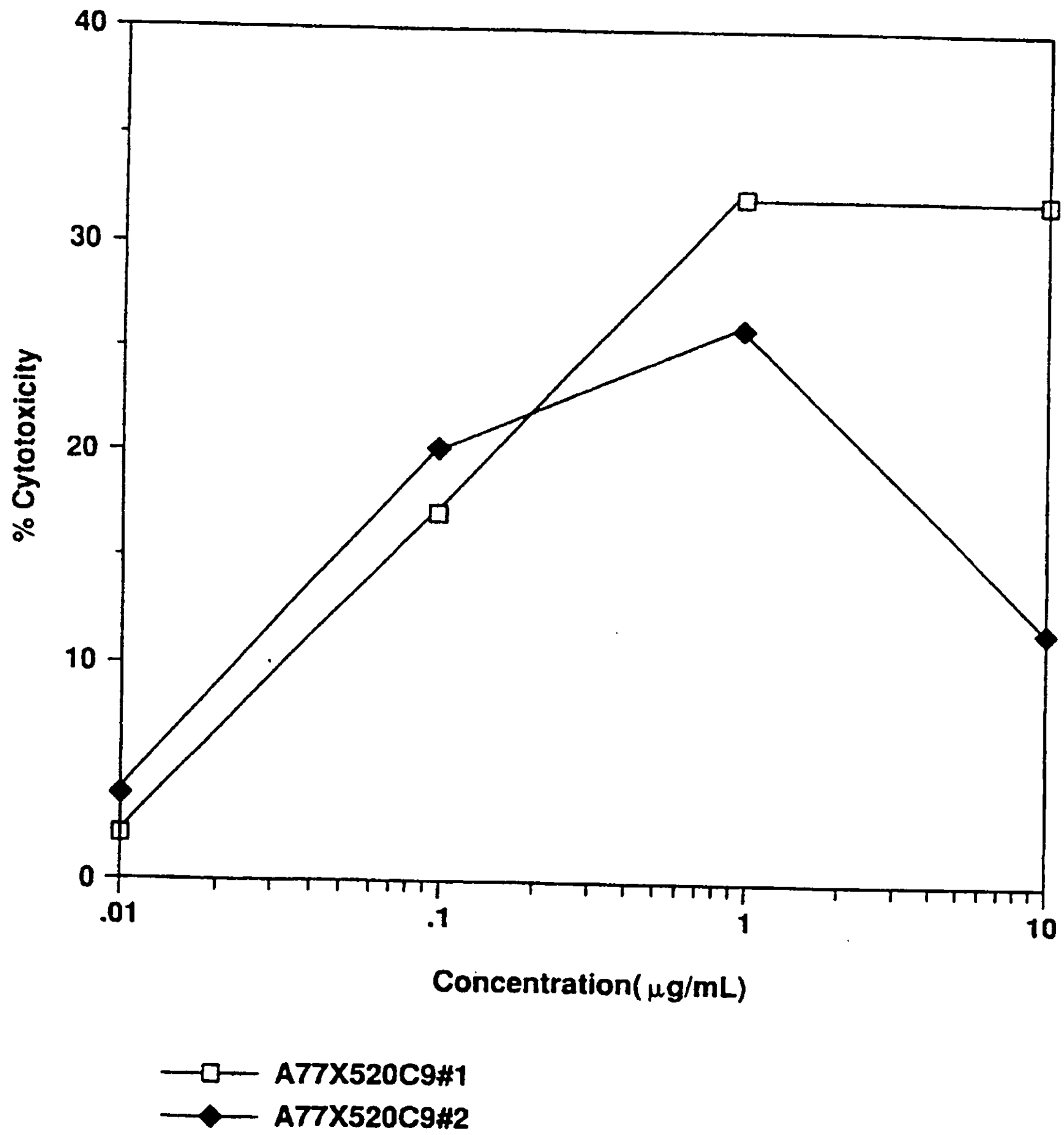

40
30
20
10
0
% Cytotoxicity
.01
.1
1
10
Concentration(μg/mL)
A77X520C9#1
A77X520C9#2

FIG.9

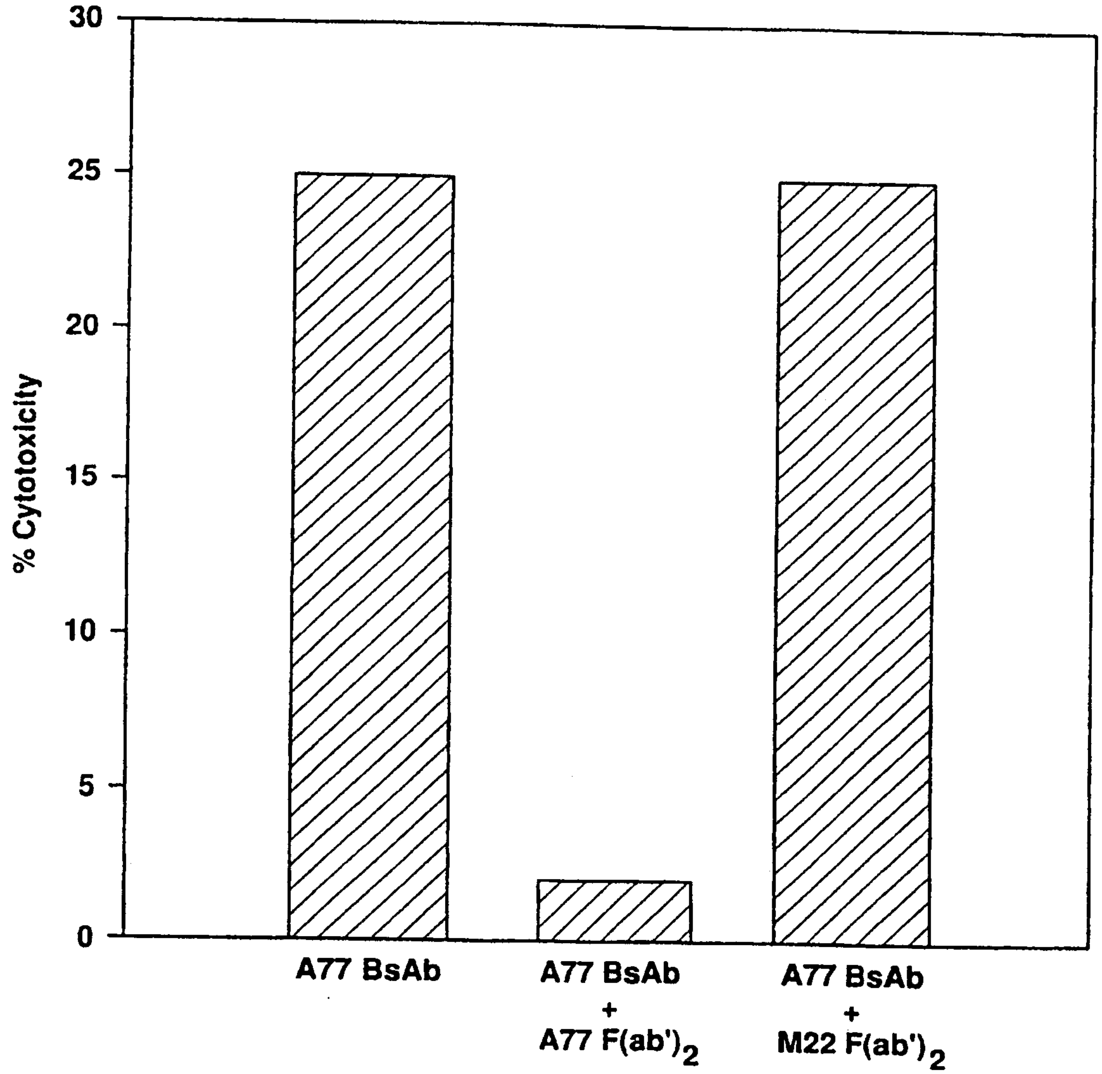

30
25
20
15
10
5
0
% Cytotoxicity
A77 BsAb
A77 BsAb + A77 F(ab')$_2$
A77 BsAb + M22 F(ab')$_2$

FIG.10

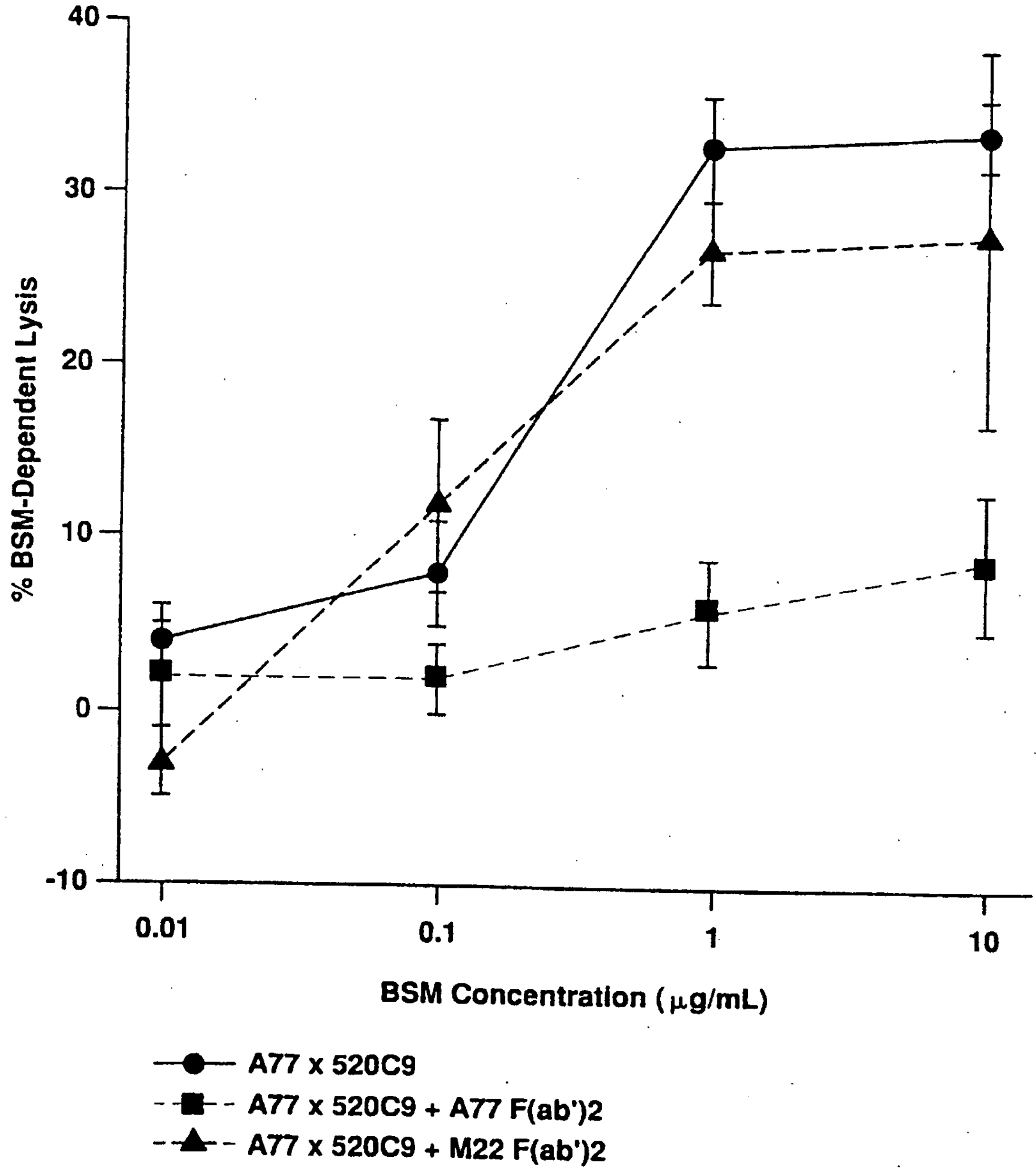

40
30
20
10
0
-10
% BSM-Dependent Lysis
0.01
0.1
1
10
BSM Concentration (μg/mL)
A77 x 520C9
A77 x 520C9 + A77 F(ab')2
A77 x 520C9 + M22 F(ab')2

FIG.11

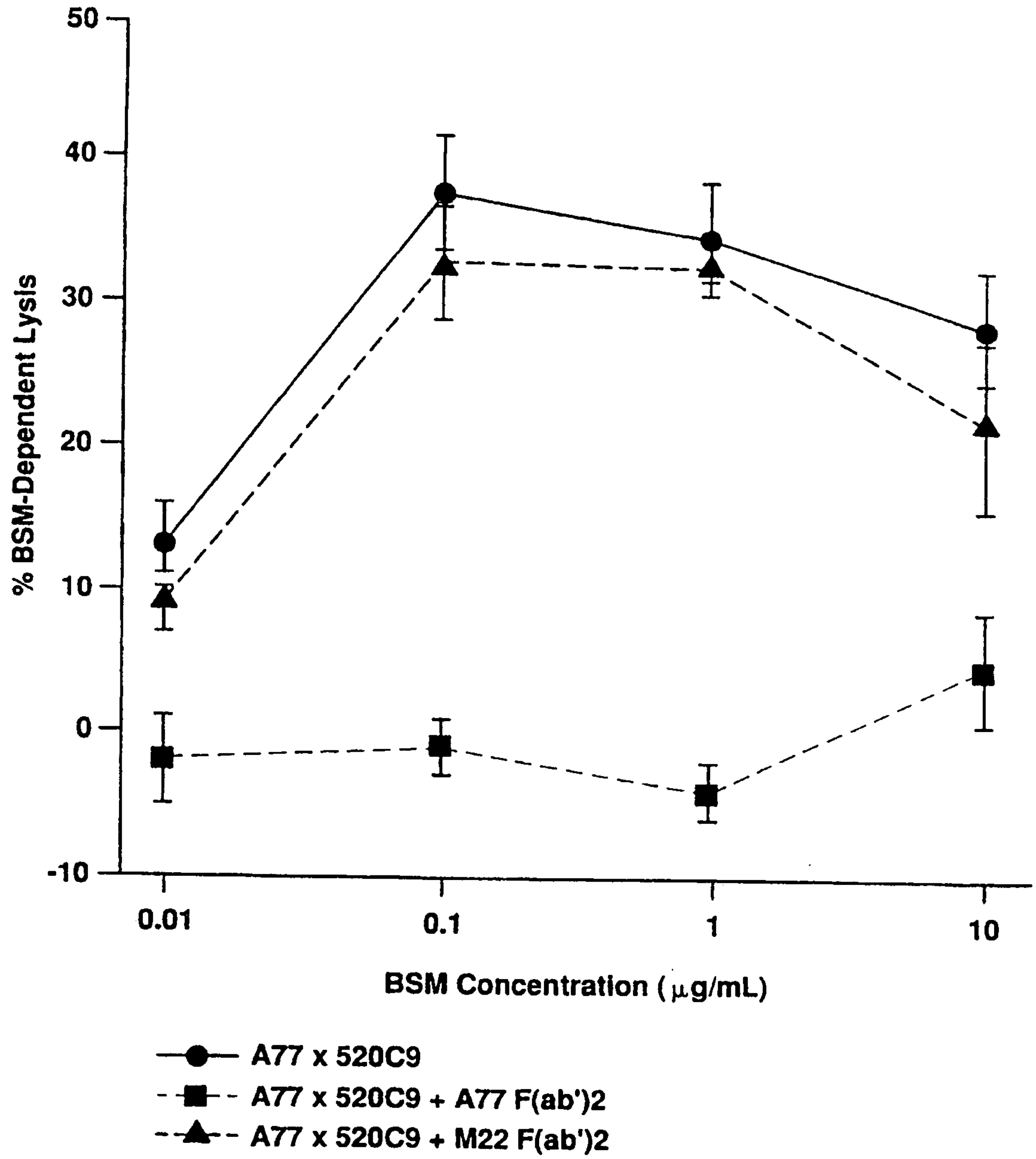

50
40
30
20
10
0
-10
% BSM-Dependent Lysis
0.01
0.1
1
10
BSM Concentration (μg/mL)
A77 x 520C9
A77 x 520C9 + A77 F(ab')2
A77 x 520C9 + M22 F(ab')2

FIG.12

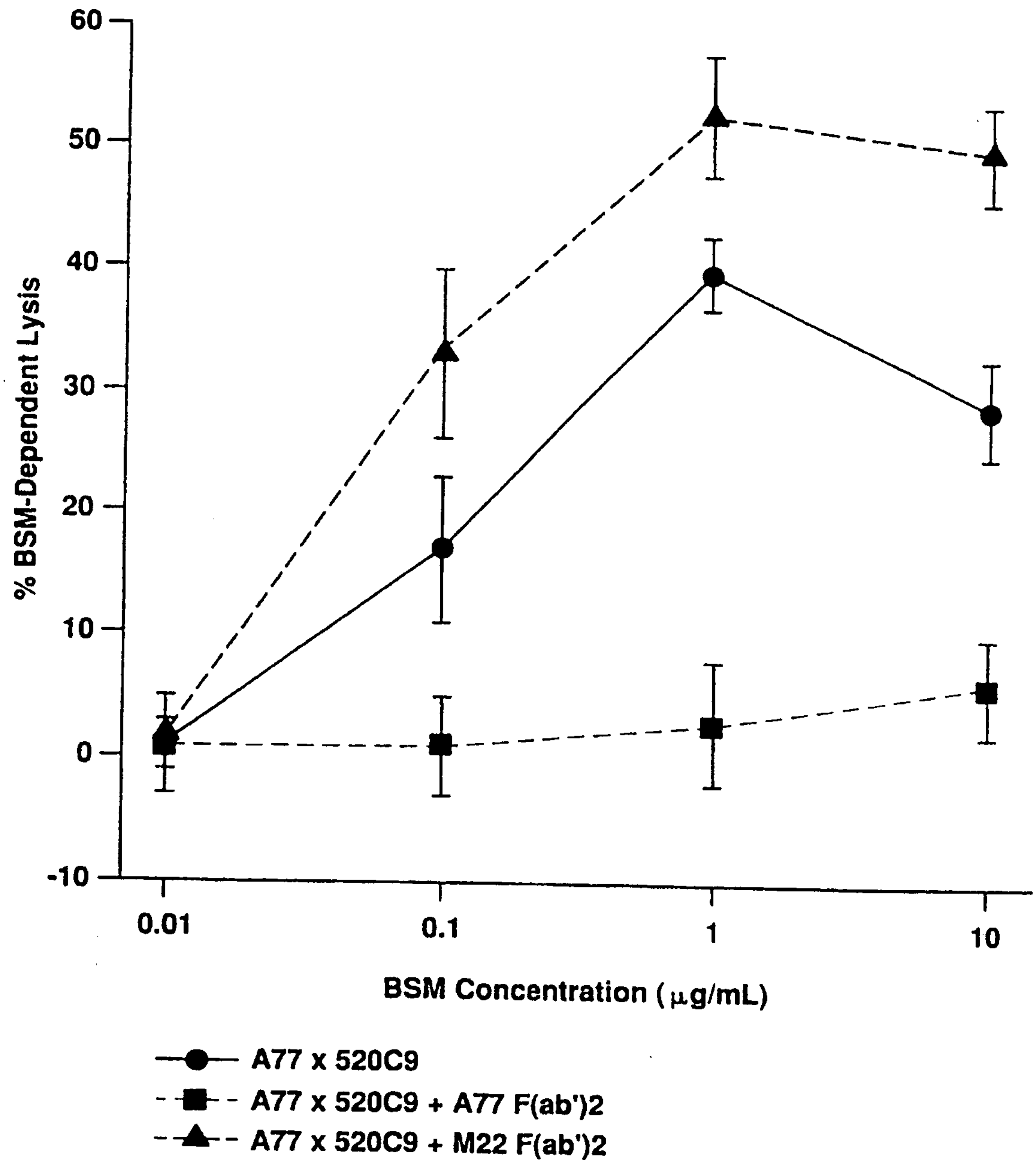

60
50
40
30
20
10
0
-10
% BSM-Dependent Lysis
0.01
0.1
1
10
BSM Concentration (μg/mL)
A77 x 520C9
A77 x 520C9 + A77 F(ab')2
A77 x 520C9 + M22 F(ab')2

FIG.13

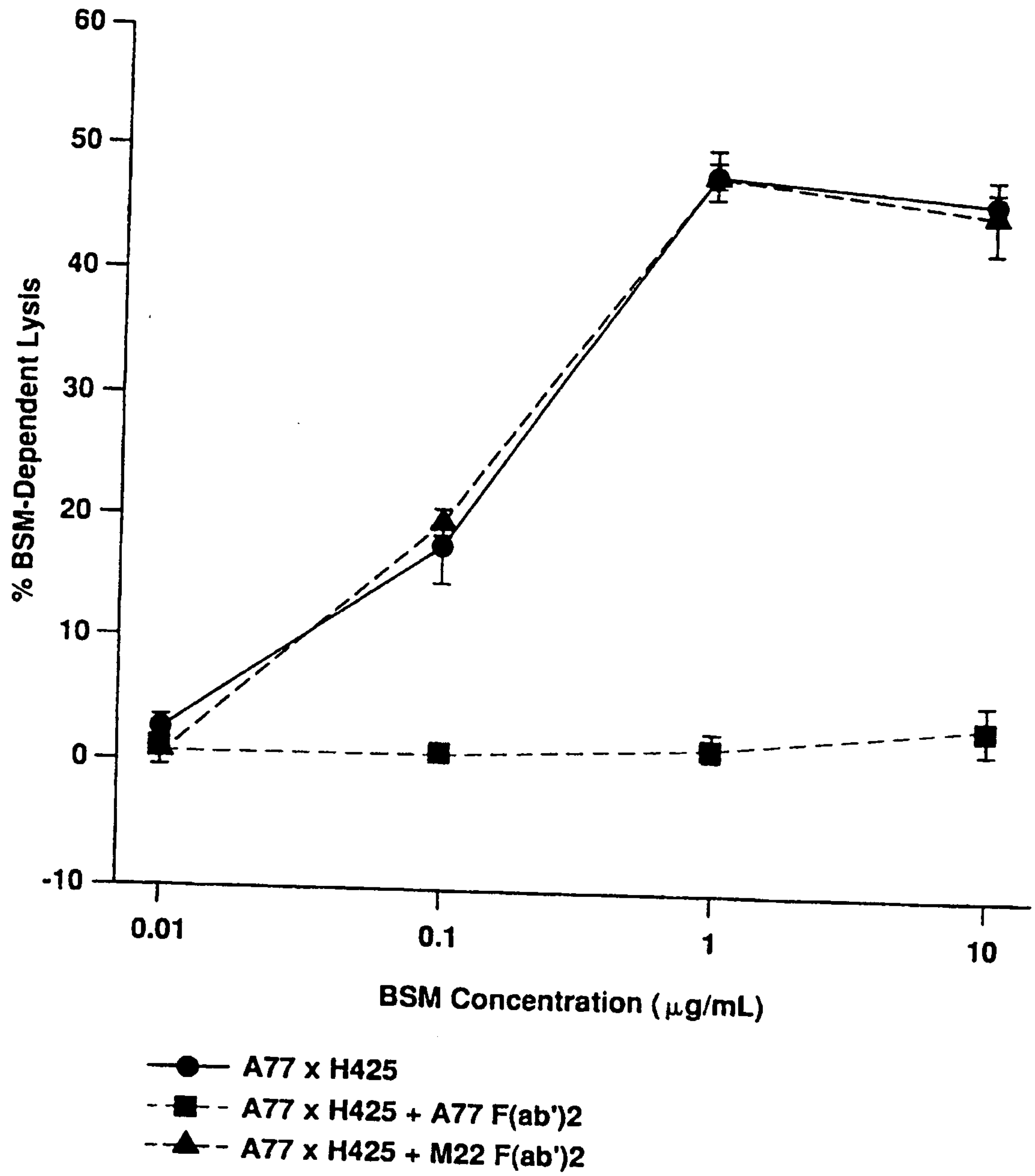

60
50
40
30
20
10
0
-10
% BSM-Dependent Lysis
0.01
0.1
1
10
BSM Concentration (μg/mL)
A77 x H425
A77 x H425 + A77 F(ab')2
A77 x H425 + M22 F(ab')2

FIG.14

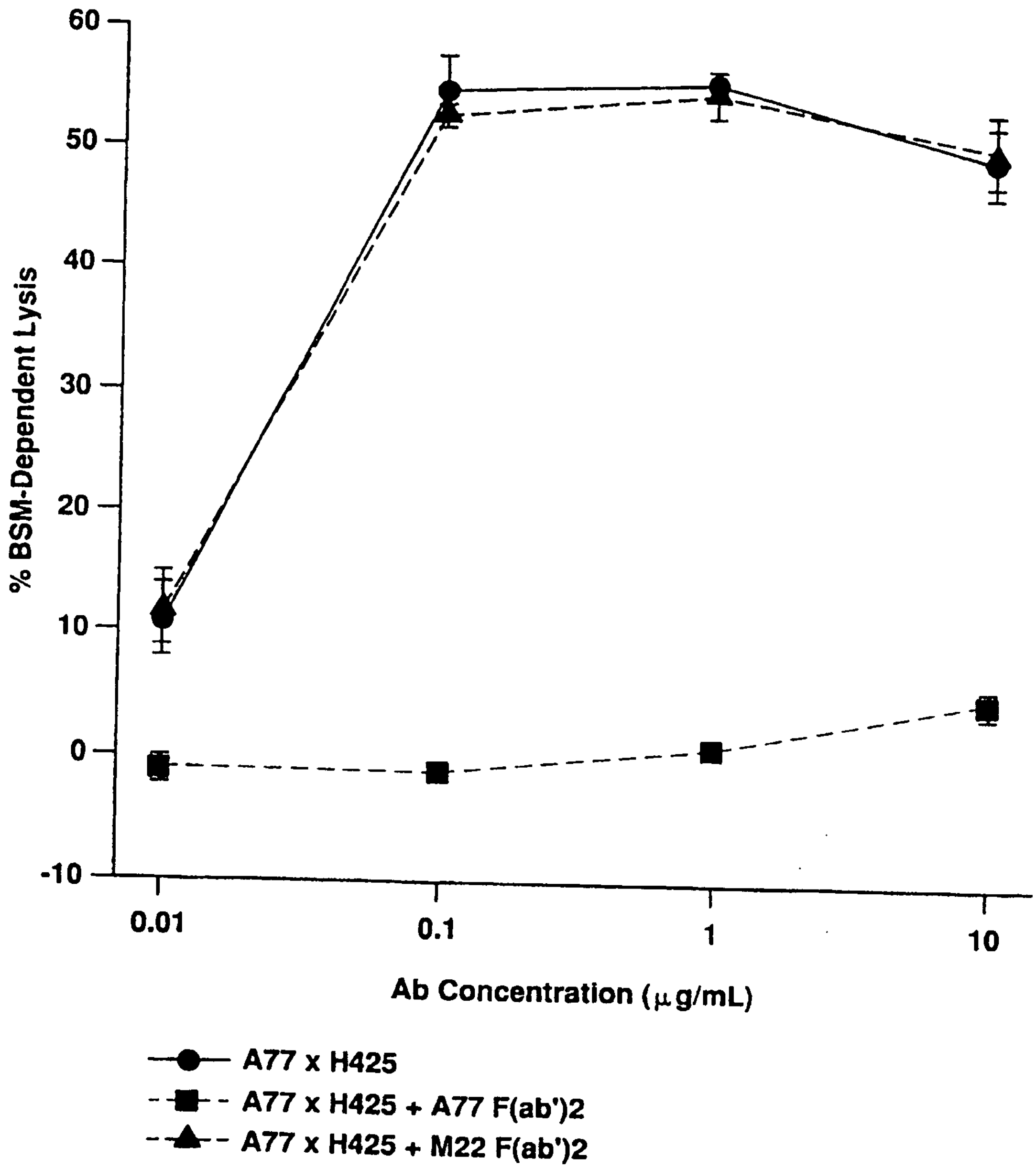

60
50
40
30
20
10
0
-10
% BSM-Dependent Lysis
0.01
0.1
1
10
Ab Concentration (μg/mL)
A77 x H425
A77 x H425 + A77 F(ab')2
A77 x H425 + M22 F(ab')2

FIG.15

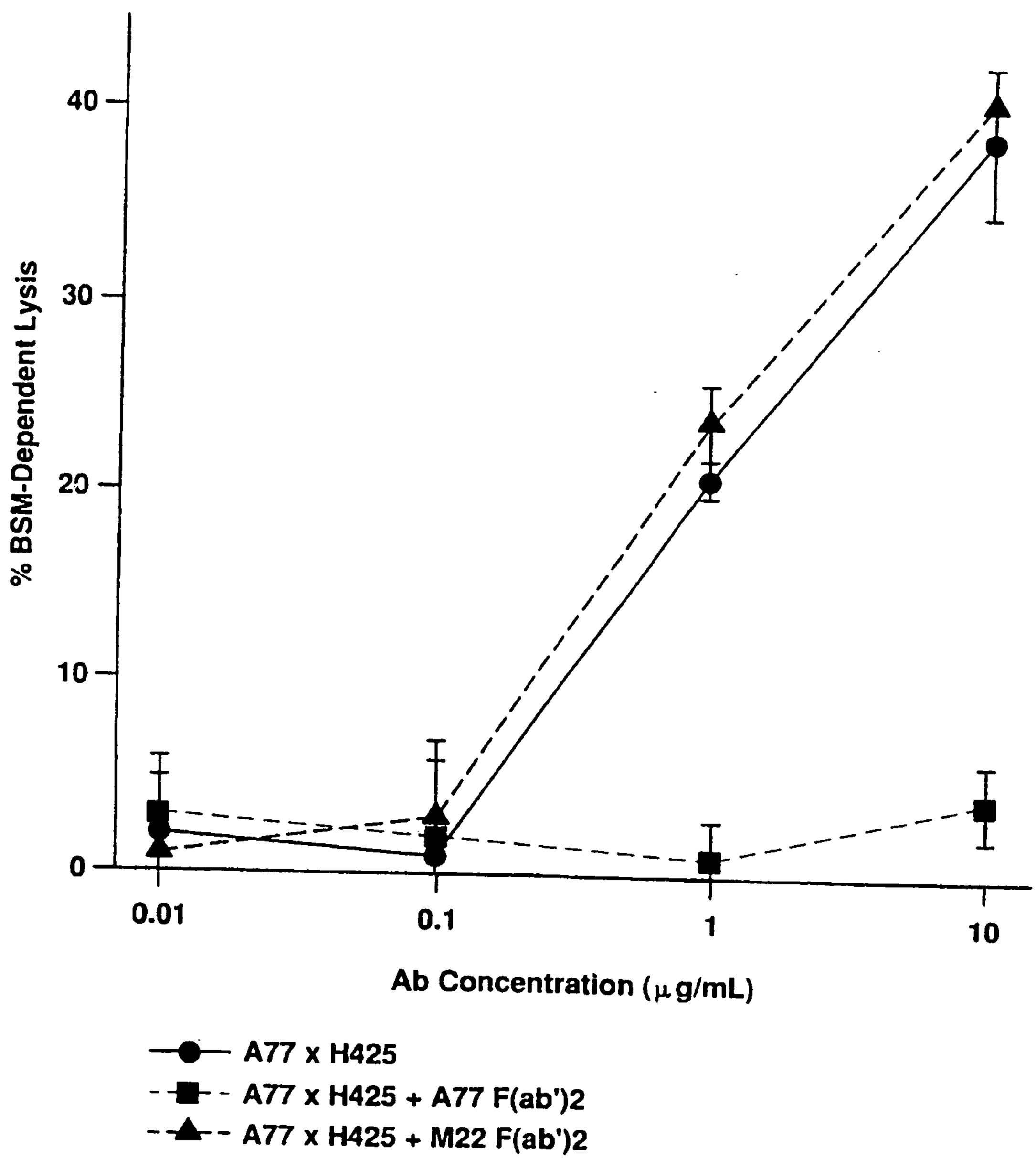

40
30
20
10
0
% BSM-Dependent Lysis
0.01
0.1
1
10
Ab Concentration (μg/mL)
A77 x H425
A77 x H425 + A77 F(ab')2
A77 x H425 + M22 F(ab')2

FIG.16

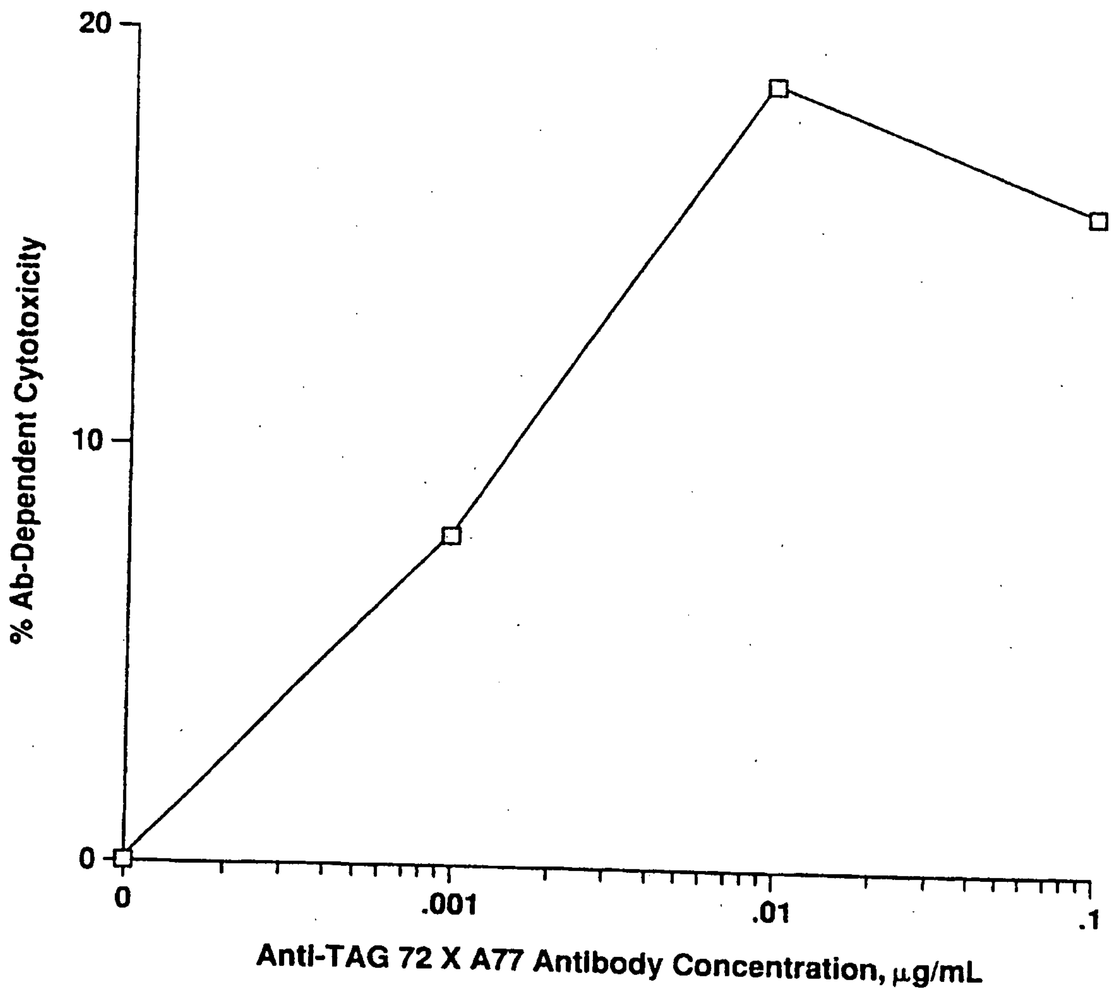

20
10
0
% Ab-Dependent Cytotoxicity
0
.001
.01
.1
Anti-TAG 72 X A77 Antibody Concentration, μg/mL

FIG.17

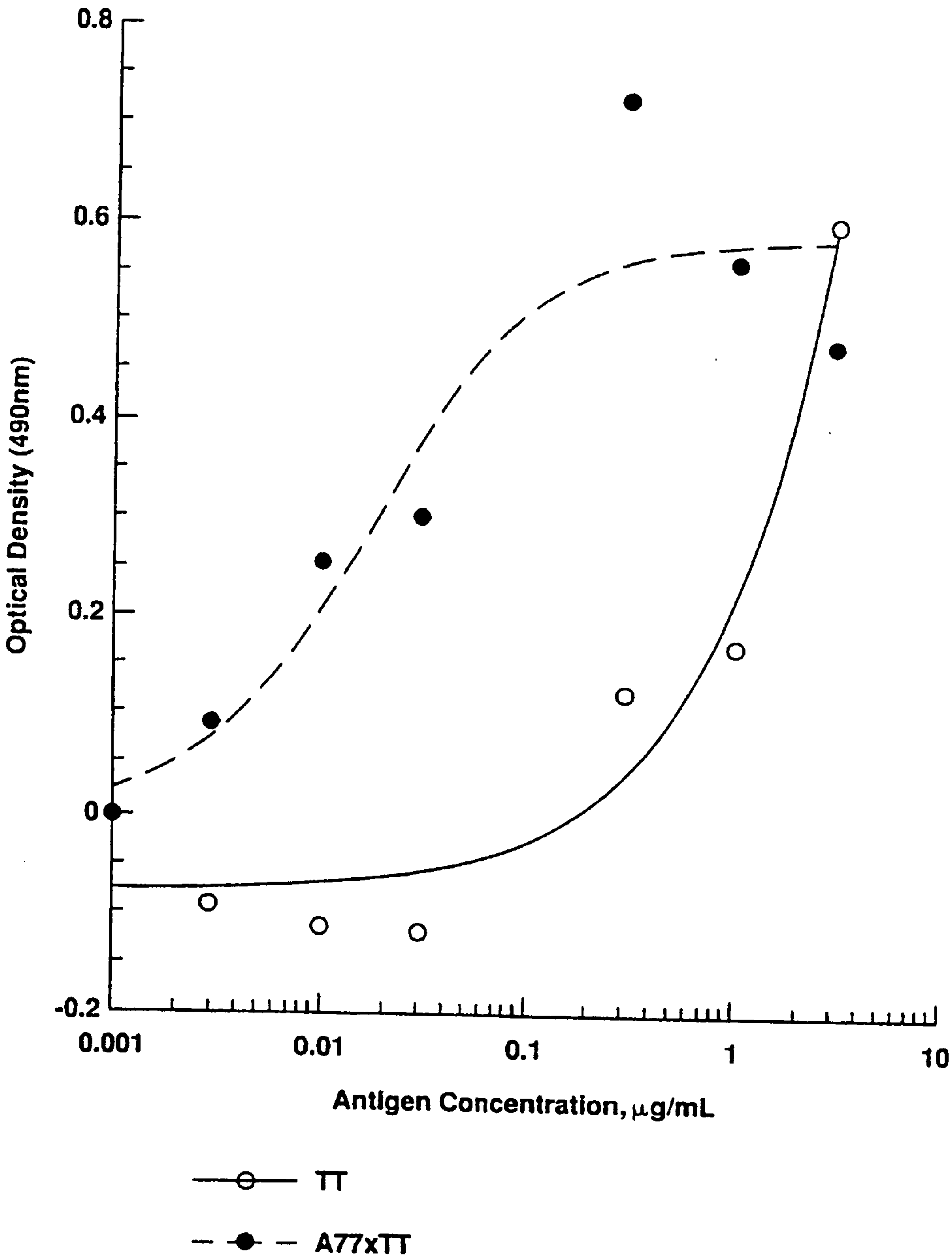

0.8
0.6
0.4
0.2
0
-0.2
Optical Density (490nm)
0.001
0.01
0.1
1
10
Antigen Concentration, µg/mL
TT
A77xTT

FIG18

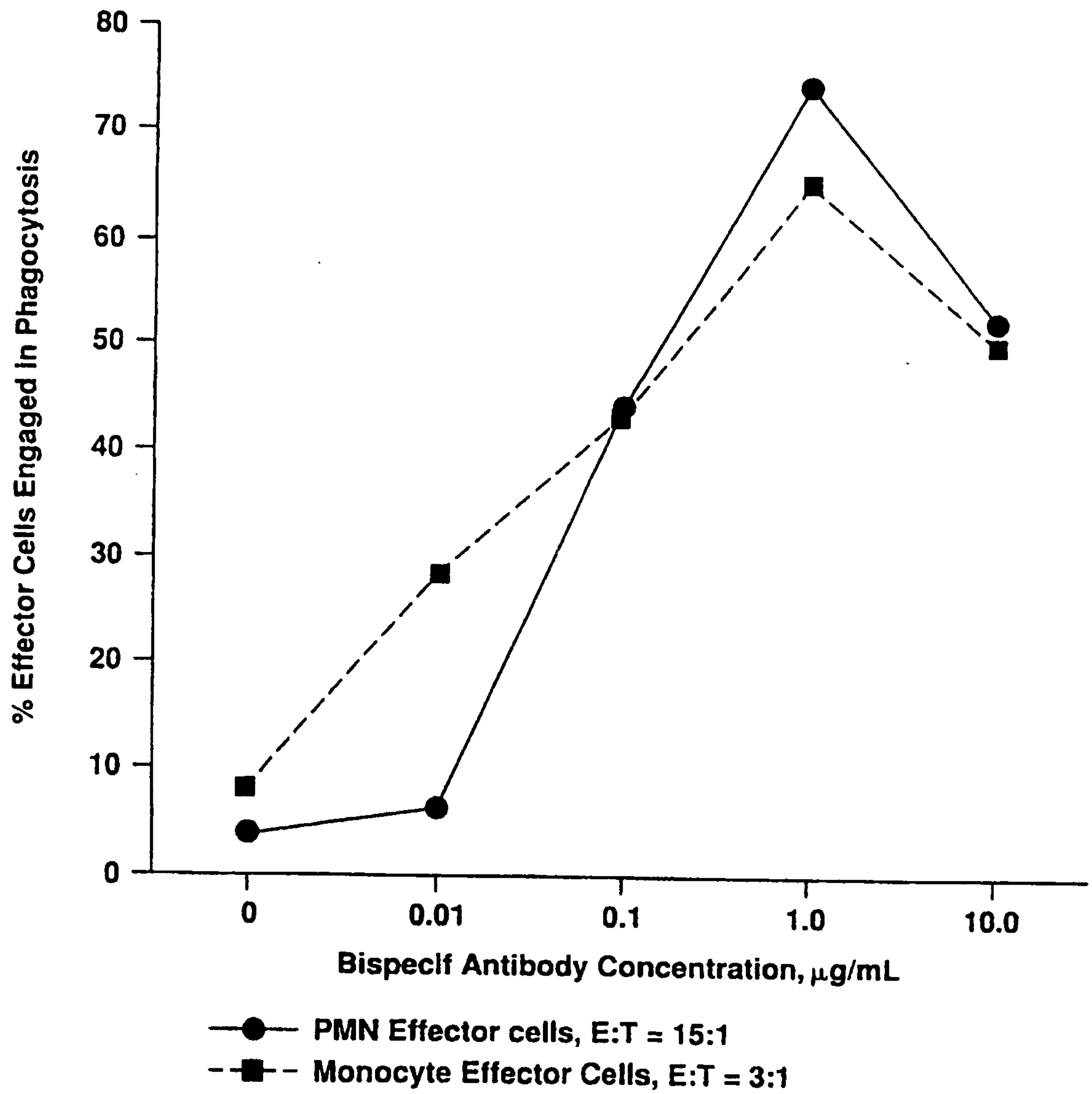

80
70
60
50
40
30
20
10
0
% Effector Cells Engaged in Phagocytosis
0
0.01
0.1
1.0
10.0
Bispecif Antibody Concentration, µg/mL
PMN Effector cells, E:T = 15:1
Monocyte Effector Cells, E:T = 3:1

FIG.19

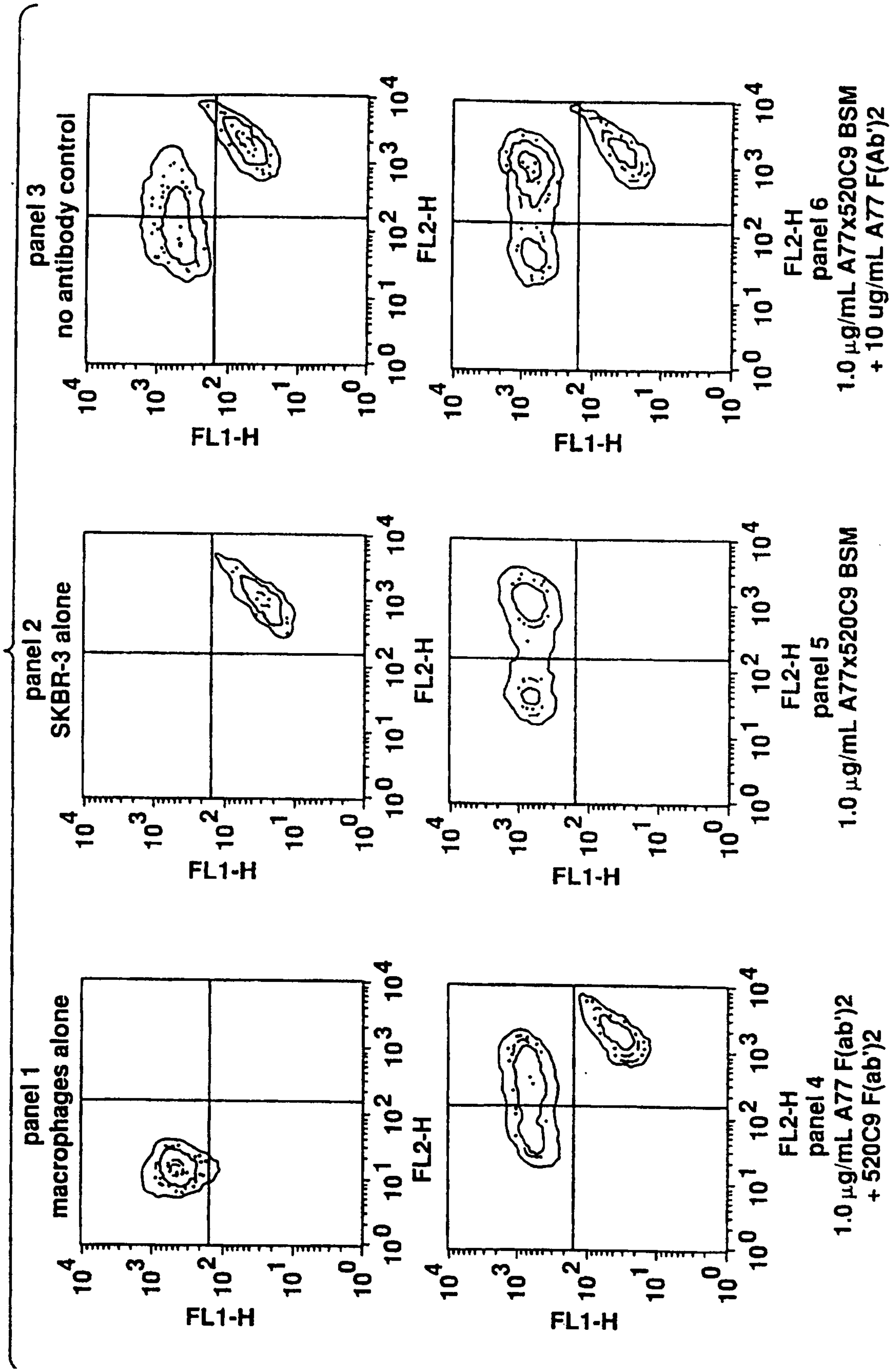

panel 1
macrophages alone
panel 2
SKBR-3 alone
panel 3
no antibody control
panel 4
1.0 µg/mL A77 F(ab')2
+ 520C9 F(ab')2
panel 5
1.0 µg/mL A77x520C9 BSM
panel 6
1.0 µg/mL A77x520C9 BSM
+ 10 ug/mL A77 F(Ab')2
FL1-H
FL2-H

FIG.20

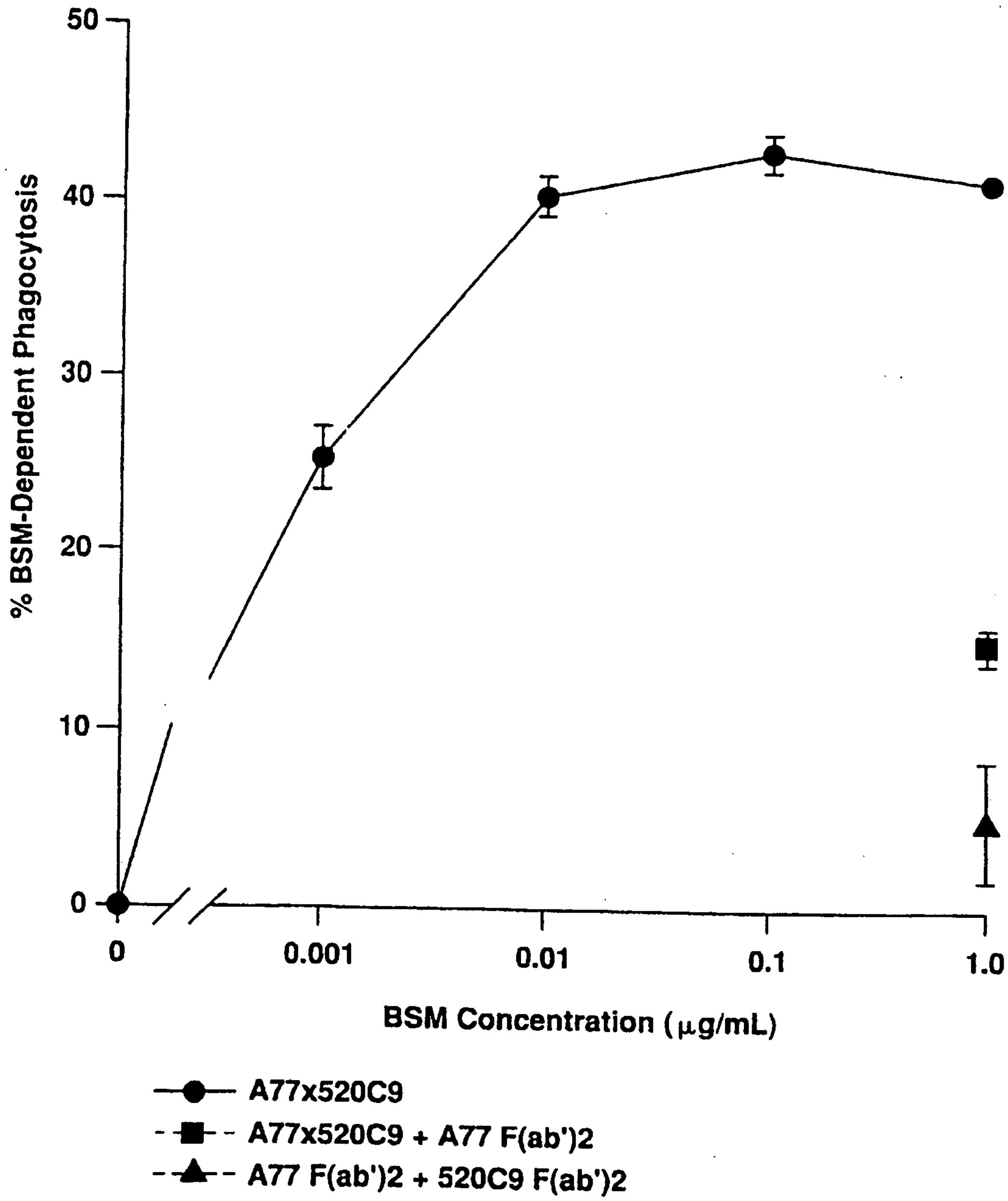

50
40
30
20
10
0
% BSM-Dependent Phagocytosis
0
0.001
0.01
0.1
1.0
BSM Concentration (μg/mL)
A77x520C9
A77x520C9 + A77 F(ab')2
A77 F(ab')2 + 520C9 F(ab')2

FIG.21A
Control

FIG.21B
[A77 x αCandida]

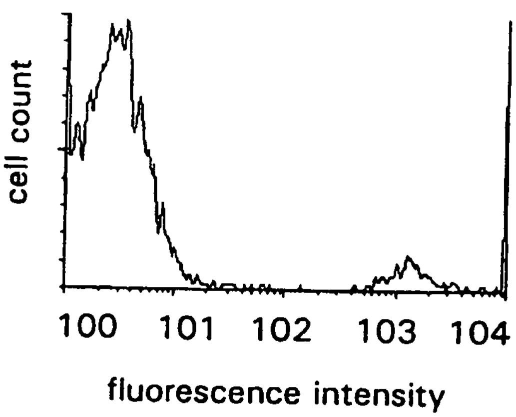


FIG.21C

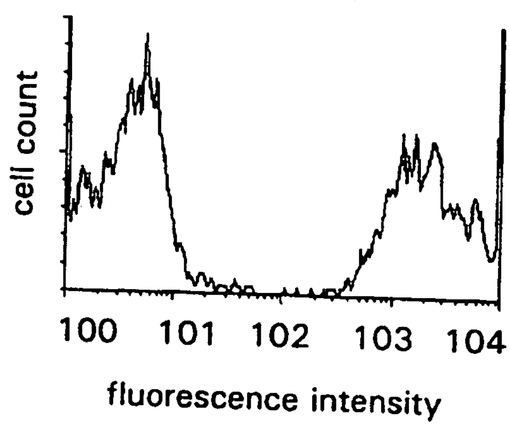


FIG.21D

FIG.22A

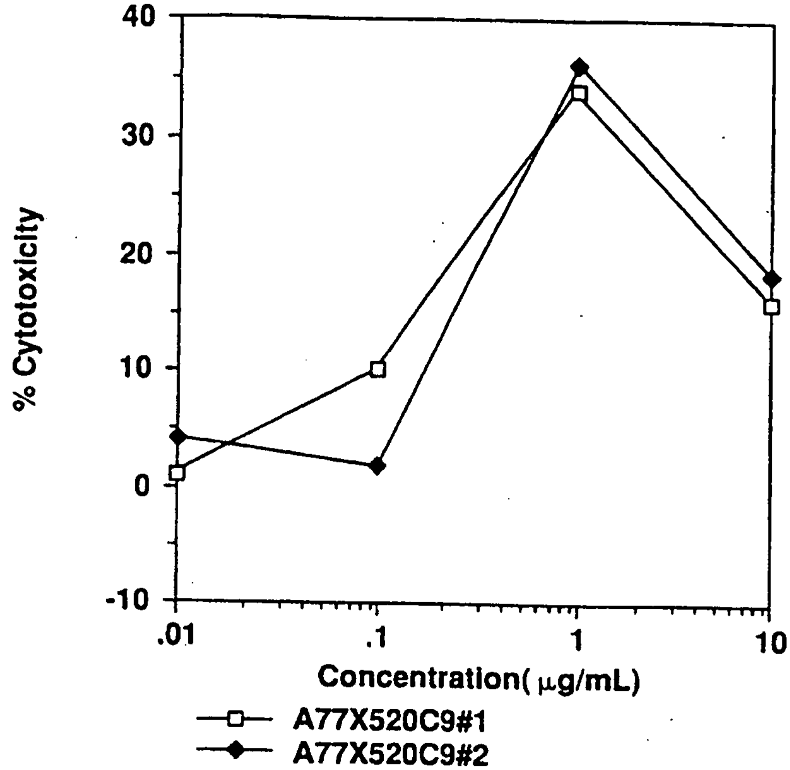
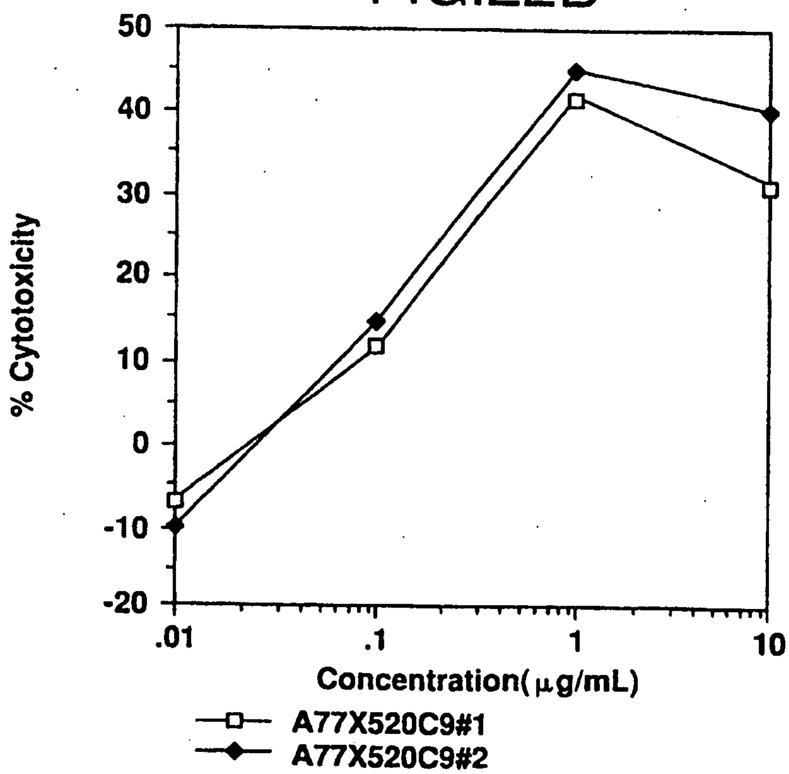


FIG.22B



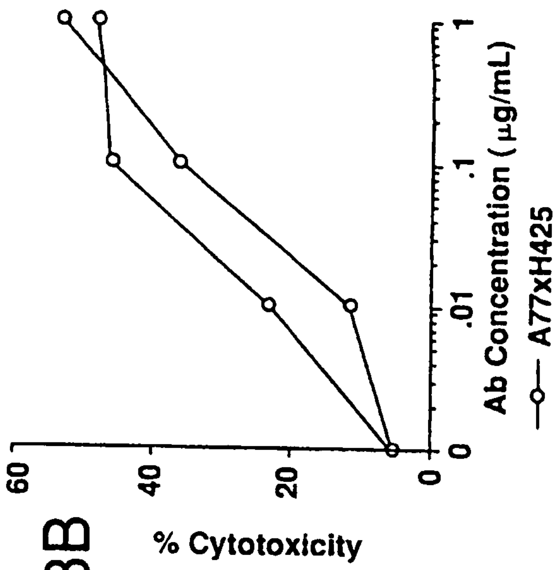


FIG.23B

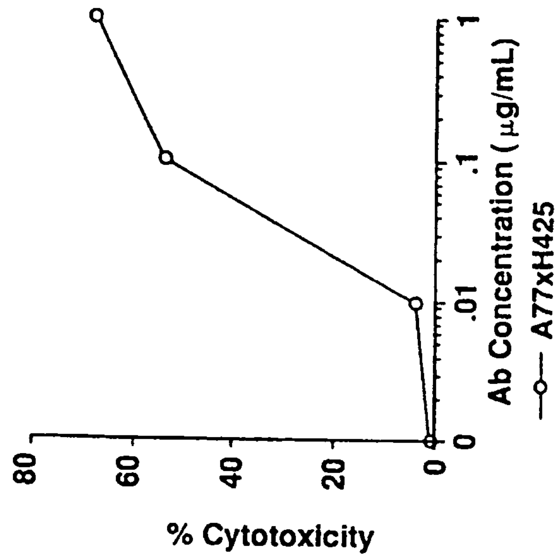


FIG.23C

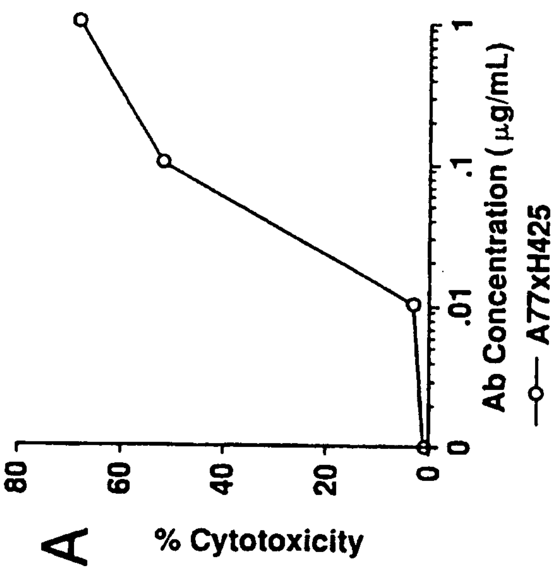


FIG.23A

FIG.24

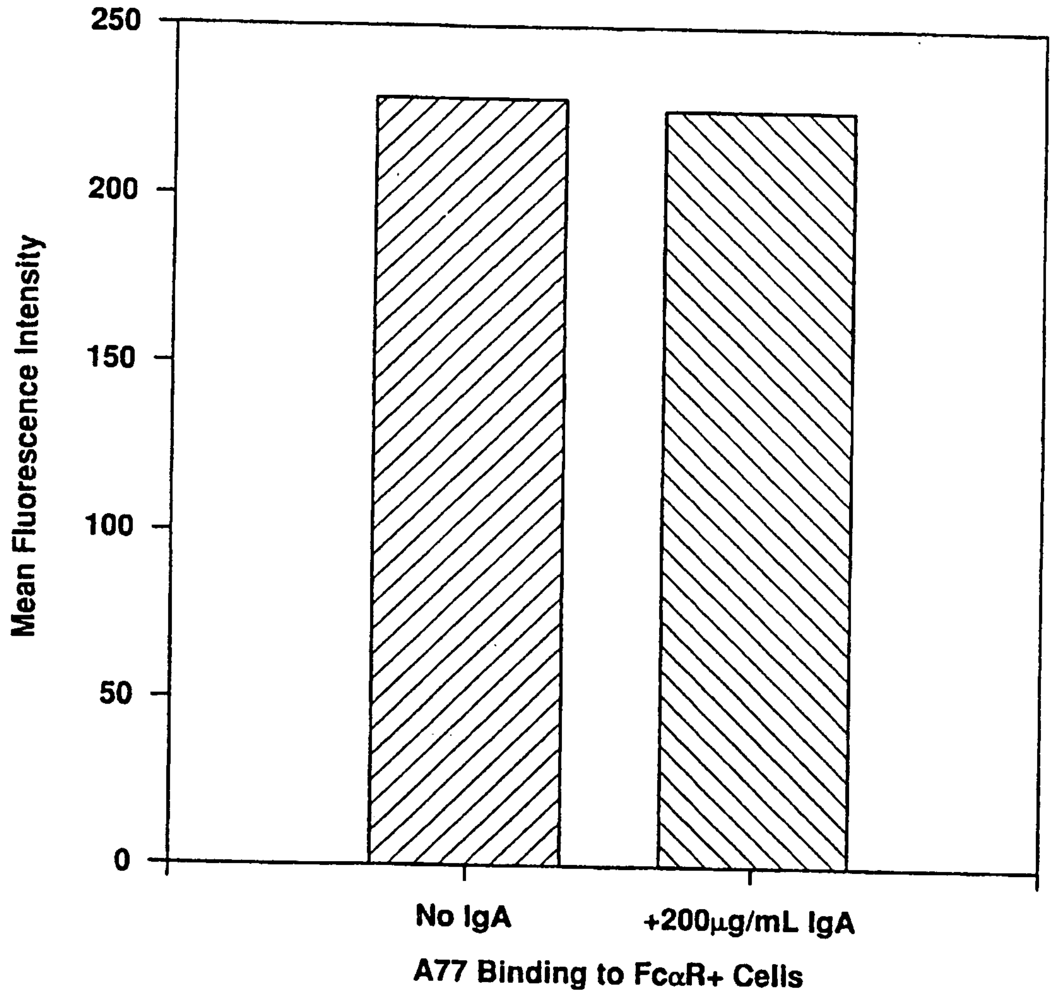

250
200
150
100
50
0
Mean Fluorescence Intensity
No IgA
+200µg/mL IgA
A77 Binding to FcαR+ Cells

FIG.25

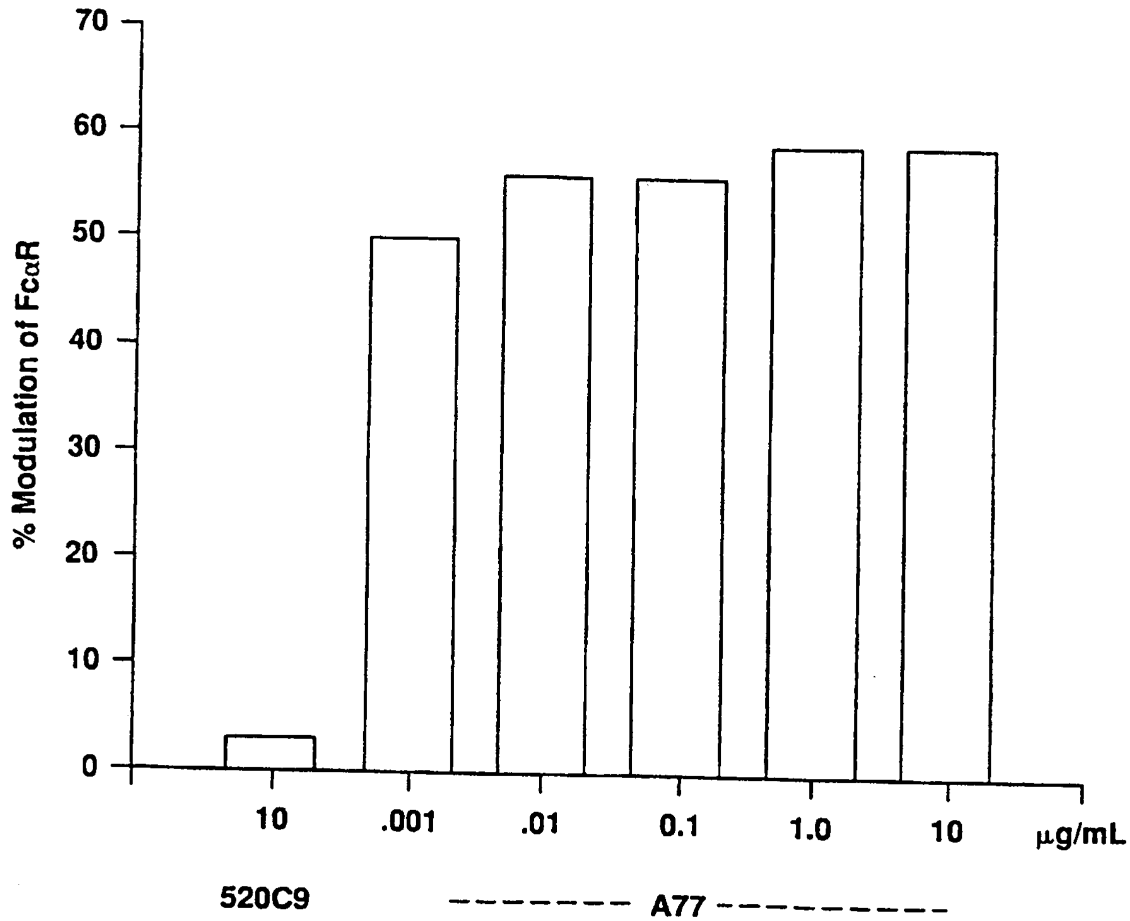

% Modulation of FcαR
70
60
50
40
30
20
10
0
10
.001
.01
0.1
1.0
10
μg/mL
520C9
-------- A77 ----------

FIG.26A

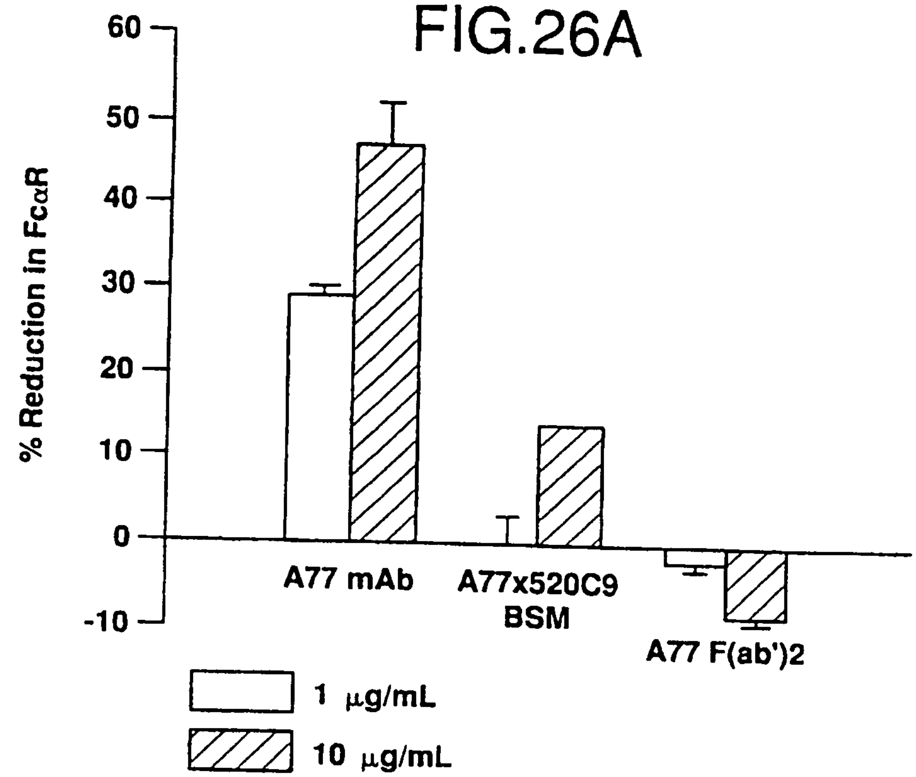
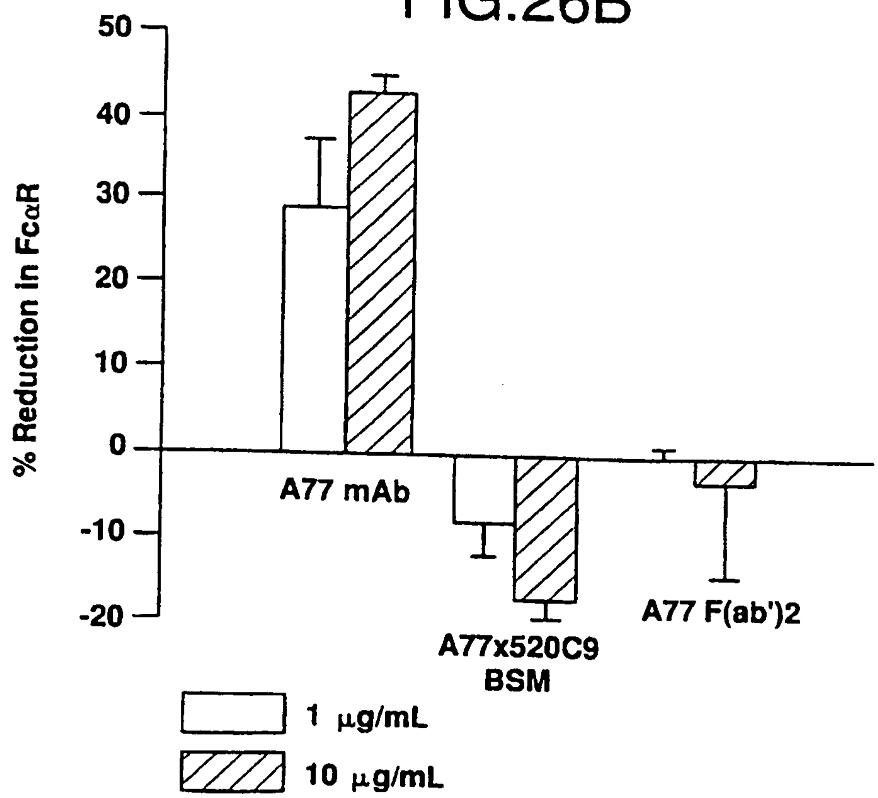


FIG.26B



```
                10            20            30              40
      *          *     *      *      *      *      *      *     *
GAC ATT CAG CTG ACC CAG TCT CCA CTC ACT TTG TCG ATT ACC ATT GGA
CTG TAA GTC GAC TGG GTC AGA GGT GAG TGA AAC AGC TAA TGG TAA CCT
Asp Ile Gln Leu Thr Gln Ser Pro Leu Thr Leu Ser Ile Thr Ile Gly>

50            60             70           80            90
 *      *      *      *      *      *      *      *      *      *
CAA CCA GCC TCC ATC TCT TGC AAG TCA AGT CAG AGC CTC TTA GAT AGT
GTT GGT CGG AGG TAG AGA ACG TTC AGT TCA GTC TCG GAG AAT CTA TCA
Gln Pro Ala Ser Ile Ser Cys Lys Ser Ser Gln Ser Leu Leu Asp Ser>

  100           110           120            130           140
    *      *      *      *      *      *      *      *      *
GAT GGA AAG ACA TAT TTG AAT TGG TTG TTA CAG AGG CCA GGC CAG TCT
CTA CCT TTC TGT ATA AAC TTA ACC AAC AAT GTC TCC GGT CCG GTC AGA
Asp Gly Lys Thr Tyr Leu Asn Trp Leu Leu Gln Arg Pro Gly Gln Ser>

     150           160           170           180           190
*      *      *      *      *      *      *      *      *      *
CCA ACG CGC CTA ATC TAT CTG GTG TCT AAA CTG GAC TCT GGA GTC CCT
GGT TGC GCG GAT TAG ATA GAC CAC AGA TTT GAC CTG AGA CCT CAG GGA
Pro Thr Arg Leu Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro>

       200           210            220           230           240
  *      *      *      *      *      *      *      *      *      *
GAC AGG TTC ACT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTG AAA ATC
CTG TCC AAG TGA CCG TCA CCT AGT CCC TGT CTA AAG TGT GAC TTT TAG
Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile>

            250           260           270           280
       *      *      *      *      *      *      *      *      *
AGC AGA GTG GAG GCT GAG GAT TTG GGA ATT TAT TAT TGC TGG CAA GGT
TCG TCT CAC CTC CGA CTC CTA AAC CCT TAA ATA ATA ACG ACC GTT CCA
Ser Arg Val Glu Ala Glu Asp Leu Gly Ile Tyr Tyr Cys Trp Gln Gly>

290           300           310           320           330
 *      *      *      *      *      *      *      *      *      *
GCA CAT TTT CCT CAG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA
CGT GTA AAA GGA GTC TGC AAG CCA CCT CCG TGG TTC GAC CTT TAG TTT
Ala His Phe Pro Gln Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys>
```

FIG.27

```
            10                  20                  30                  40
     *       *        *        *        *         *        *        *        *
ATG GGA TGG AGC TGG GTC ATT ATC TTC CTC CTG TCA GGA ACT GCA GGA
TAC CCT ACC TCG ACC CAG TAA TAG AAG GAG GAC AGT CCT TGA CGT CCT
Met Gly Trp Ser Trp Val Ile Ile Phe Leu Leu Ser Gly Thr Ala Gly>

50                60                  70                  80                  90
 *        *        *        *        *        *        *        *        *        *
GCC CAC TCT GAG ATC CAG CTG CAG CAG ACT GGA CCT GAG CTG GTG AAG
CGG GTG AGA CTC TAG GTC GAC GTC GTC TGA CCT GGA CTC GAC CAC TTC
Ala His Ser Glu Ile Gln Leu Gln Gln Thr Gly Pro Glu Leu Val Lys>

   100               110                 120                130                140
    *        *        *        *        *        *        *        *        *
CCT GGG GCT TCA GTG AAG ATA TCC TGC AAG GCT TCT GGT TAT TCA TTC
GGA CCC CGA AGT CAC TTC TAT AGG ACG TTC CGA AGA CCA ATA AGT AAG
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe>

      150                 160                 170                180                 190
 *        *        *        *        *        *        *        *        *        *
ACT GAC TAC ATC ATA TTT TGG GTG AAG CAG AGC CAT GGA AAG AGC CTT
TGA CTG ATG TAG TAT AAA ACC CAC TTC GTC TCG GTA CCT TTC TCG GAA
Thr Asp Tyr Ile Ile Phe Trp Val Lys Gln Ser His Gly Lys Ser Leu>

          200                210                 220                230                240
    *        *        *        *        *        *        *        *        *        *
GAG TGG ACT GGA AAT ATT AAT CCT TAC TAT GGT AGT ACT AGC TAC AAT
CTC ACC TGA CCT TTA TAA TTA GGA ATG ATA CCA TCA TGA TCG ATG TTA
Glu Trp Thr Gly Asn Ile Asn Pro Tyr Tyr Gly Ser Thr Ser Tyr Asn>

              250                260                 270                280
       *        *        *        *        *        *        *        *        *
CTG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA GAC AAA TCT TCC AGC
GAC TTC AAG TTC CCG TTC CGG TGT AAC TGA CAT CTG TTT AGA AGG TCG
Leu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser>

290               300                 310                 320                330
 *        *        *        *        *        *        *        *        *        *
ACA GCC TAC ATG CAG CTC AAC AGT CTG ACA TCT GAG GAC TCT GCA GTC
TGT CGG ATG TAC GTC GAG TTG TCA GAC TGT AGA CTC CTG AGA CGT CAG
Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val>

   340                350                 360                370                380
       *        *        *        *        *        *        *        *        *
TAT TAC TGT GTA AGA GGA GTT TAT TAC TAC GGT AGT AGC TAC GAG GCG
ATA ATG ACA CAT TCT CCT CAA ATA ATG ATG CCA TCA TCG ATG CTC CGC
Tyr Tyr Cys Val Arg Gly Val Tyr Tyr Tyr Gly Ser Ser Tyr Glu Ala>

      390                400                 410                420
 *        *        *        *        *        *        *        *        *
TTT CCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA
AAA GGA ATG ACC CCG GTT CCC TGA GAC CAG TGA CAG AGA CGT
Phe Pro Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala>
```

FIG.28